

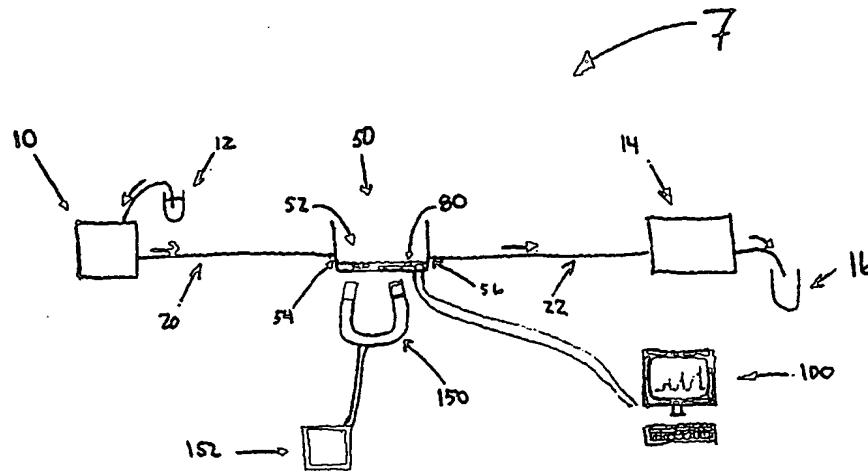
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(54) Title: ENZYME-LINKED IMMUNO-MAGNETIC ELECTROCHEMICAL BIOSENSOR



(57) Abstract

A electrochemical biosensor system based on enzyme-linked immuno-magnetic sandwich assay wherein an interdigitated array of electrodes is equipped with a magnet to attract magnetic beads. Magnetic particles bear a first recognition molecule capable of binding to the analyte. Enzymes are chemically modified to complex with the analyte. When the sandwich assay is performed, a substrate is added. The substrate is chosen such that it is cleavable by the enzyme in a reporting molecule capable of redox recycling. A substrate when cleaved preferably leads to a p-aminophenol.

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DESCRIPTION

ENZYME-LINKED IMMUNO-MAGNETIC ELECTROCHEMICAL BIOSENSOR

FIELD OF THE INVENTION

The present invention relates to devices and methods for detecting and quantitating specific analytes in a sample.

5

BACKGROUND OF THE INVENTION

The detection and quantitation of specific analytes in a sample is an important activity in environmental, health, biotechnology, industrial chemistry and other fields. The 10 assays have also found use in high throughput screening, screening of oligo libraries in the field of functional genomics analysis, combinatorial chemistry screening, and other such fields. The analytes detected or quantitated may be any compound of interest for which there is a specific 15 recognition molecule. Well known recognition molecules include proteins, such as receptors, immuno-globulins, and the like, nucleic acids, their analogs, and the like, haptens, hormones, polypeptides, certain drugs and other such molecules.

20 Devices and techniques for detecting analytes are well known in the art. These including ELISAs, RIAs, PCR, and the like. Although these techniques have proven very powerful, effective and valuable, they suffer from drawbacks.

25 Most devices and techniques presently used for the detection of analytes require relatively long reaction

times, complex processes and laboratory conditions. For example, temperatures above room temperature, reaction times in excess of 30 minutes, and strict time limitations. Other drawbacks have included auto-fluorescence of reagents or 5 analytes, in particular in the field of combinatorial chemistry, and when screening small peptide libraries using optical methods.

Decreasing the time necessary to perform an assay while maintaining the precision, sensitivity, reliability and 10 dose-dependent results that can be obtained using conventional methods presents great economic advantages, and the patient's well-being in the case of laboratory medicine. The use of an electrochemical sensor rather than an optical sensor further presents other advantages, including avoiding 15 auto-fluorescence and turbidity problems.

SUMMARY OF THE INVENTION

In a first, independent aspect of the present invention, an electrochemical sensor includes an 20 interdigitated array of electrodes on a substantially dielectric substrate and a means for concentrating reagents on the surface of the interdigitated array of electrodes.

In a second, independent aspect of the present invention, an electrochemical reporter system includes a 25 first recognition molecule linked to a magnetic bead, wherein the first recognition molecule can specifically bind an analyte; a second recognition molecule linked to an enzyme, for coupling with specificity the enzyme to the analyte or the first recognition molecule/analyte complex; a 30 substrate, which in the presence of the enzyme is processed into an electrochemical reporter molecule capable of redox recycling; a sensor for detecting the electrochemical

reporter molecule, wherein the sensor has a configuration such that electrochemical reporter molecules, if present, exhibit redox recycling; and a magnetic field generating device positioned such that the magnetic field it generates 5 can attract to the surface of the sensor magnetic beads in solution over the sensor.

In a third, independent aspect of the present invention, an electrochemical reporter device includes a chamber for receiving an analytical reaction having magnetic 10 beads; a sensor on a surface of the chamber, the sensor for detecting electrochemical reporter molecules within the chamber, the sensor having a configuration such that it causes redox recycling of reporter molecules capable of exhibiting redox recycling; and a magnetic field generating 15 device capable of generating a magnetic field that attracts magnetic beads present within the chamber onto the sensor.

In a fourth, independent aspect of the present invention, an electrochemical reporter system includes a magnetic bead; a first recognition molecule capable of 20 specifically binding an analyte, the first recognition molecule being linked to the magnetic bead; an enzyme; a coupling element, or second recognition molecule, for coupling with specificity the enzyme to the analyte or the first recognition molecule/analyte complex; a substrate, 25 which in the presence of the enzyme is cleavable into an electrochemical reporter molecule capable of exhibiting redox recycling; a sensor for detecting the electrochemical reporter molecule and having a configuration such that the reporter molecule will exhibit redox recycling; and a 30 magnetic field generating device positioned such that the magnetic beads may be attracted to the vicinity of the sensor.

In a fifth, independent aspect of the present invention, an assay for detecting or quantitating a specific analyte in a sample comprises the following steps: a primary incubation, wherein magnetic beads coated with a recognition molecule that specifically binds an analyte are incubated with a sample; a secondary incubation, wherein the magnetic beads are then incubated with a conjugate comprising an enzyme and a molecule that specifically binds the analyte, or the analyte/recognition molecule complex; capturing the magnetic beads with a magnetic field generating device over a sensor capable of producing redox recycling of an electrochemical capable of undergoing redox recycling; adding a substrate, wherein the substrate in the presence of the enzyme is cleaved into an electrochemical reporter molecule capable of undergoing redox recycling; detecting the presence or measuring the amount of electrochemical present in the solution with the sensor.

In a sixth, independent aspect of the present invention, an electrochemical immunoassay for detecting an analyte in a sample includes the steps of providing an antigen linked to a magnetic bead and an antibody specific for an analyte bound to the antigen, wherein the antibody is coupled to an enzyme or has a coupling element such that it can be specifically coupled to an enzyme; contacting the magnetic bead/antigen/antibody/enzyme complex with a sample to be analyzed; attracting the magnetic bead/antigen/antibody/enzyme complex to the vicinity of a sensor; adding a substrate to the collected magnetic bead/antigen/antibody/enzyme complex, wherein the substrate in the presence of the enzyme is cleaved into an electrochemical reporter molecule capable of exhibiting redox recycling; detecting the presence or measuring the

amount of reporter molecule with the sensor, wherein the sensor is an interdigitated array of electrodes capable of producing redox recycling of the reporter molecule.

In a seventh, independent aspect of the present invention, an electrochemical immunoassay for detecting a specific analyte in a sample includes the steps of providing a recognition molecule linked to a magnetic bead, wherein the recognition molecule is capable of specifically binding the analyte; contacting the magnetic bead with a sample to be analyzed; coupling with specificity an enzyme to the analyte or the recognition molecule/analyte complex; attracting the magnetic bead/recognition molecule/analyte/coupling element-enzyme complex to the vicinity of a sensor with a device capable of generating a magnetic field; adding a substrate, which in the presence of the enzyme is cleaved into an electrochemical reporter molecule capable of exhibiting redox recycling; detecting the presence or measuring the amount of electrochemical with the sensor, wherein the sensor is an interdigitated array of electrodes capable of producing redox recycling of the electrochemical reporter molecule.

In an eighth, independent aspect of the present invention, an electrochemical reporter system includes a magnetic bead; a recognition molecule capable of specifically binding an analyte, the recognition molecule being linked to the magnetic bead; an enzyme; a coupling element, for coupling with specificity the enzyme to the analyte or recognition molecule/analyte complex; a substrate, which in the presence of the enzyme is cleavable into a reporter molecule capable of exhibiting redox recycling; a sensor, for detecting the electrochemical reporter molecule and having a configuration such that the

reporter molecule will exhibit redox recycling; a magnetic field generating device positioned such that the magnetic beads will be attracted to the vicinity of the sensor.

5 BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a schematic representation of a device in accordance with the present invention for a detecting and/or quantitating a specific analyte in a sample.

10 FIG. 2 is a graphic representation of the change in current measured when assaying in accordance with the present invention serum samples having low, medium and high anti-p24 levels.

15 FIG. 3 is a graph plotting the slope of the kinetic measurement (nA/s) against the original concentration (mIU/ml) of HBsAg in the sample.

FIG. 4 is a dose response curve using electrochemical measurement according to the present invention.

20 FIG. 5 are measurements of different concentrations using electrochemical measurement with a device without the magnetic beads and magnet of the present invention.

FIG. 6 is a comparison of assays using different number of magnetic beads per sample.

25 FIG. 7 is a flow chart of methods and devices in accordance with the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Turning in detail to the drawings, FIG. 1 is a schematic representation of a device 7 in accordance with the present invention for detecting and/or quantitating a specific analyte in a sample.

A first pump 10 pumps a processed sample 12 through an inflow tubing, or first tubing segment 20, into the

electrochemical sensing module 50. The first pumping device 10 may be any device that can move fluids containing small particles, or a slurry, through a tubing segment. Preferably, the first pumping device 10 is an adjustable speed peristaltic pump.

The first tubing segment 20 may be any tubing that can carry a fluid having small particles without clogging, or transport a fluid/particulate slurry. It is preferably made from an inert material, i.e., a material that will not interact detrimentally with the fluids and reagents flowing within it. Most preferably, the first tubing segment 20 is TYGON tubing.

The diameter of the first tubing segment 20 will depend on the rate of flow desired. When the first pumping device 10 is a peristaltic pump, the rate of flow of fluids through the tube 20 can be increased by using tubing having a wider inner diameter, or by increasing the speed of the peristaltic pump. Conversely, a slower rate of flow can be achieved by using tubing having a smaller diameter or by decreasing the speed of the peristaltic pump. Preferably the first tubing segment has an inner diameter such that an appropriate rate of flow may be achieved for the specific pump being used. The diameter of the tubing used will also be a function of the size of the beads and the fluid being delivered.

The electrochemical sensing module 50, includes an inflow orifice 54, a chamber 52 for holding the sample, an outflow orifice 56 and a sensor 80. The outflow orifice 56 is connected to the outflow tubing, or second tubing segment 22. The second tubing segment 22 may be connected at its other end to a second pumping (drawing) device 14 (shown in the figure), or alternatively the first pumping device 10

may also be used for this purpose, in which case the system preferably is hermetically sealed.

The second tubing segment 22 advantageously terminates at a waste receptacle 16, collecting chamber, or the like.

5 The properties of the outflow tubing 22 are preferably the same as the inflow tubing 20, described *supra*. If two pumps are used, less flexible, more inert material can be used for the tubing, including TEFLON, or the like.

10 The electrochemical sensing module 50 may be a disposable, single use unit, in which case the module 50 preferably is adapted to slide and/or snap into and out of the device 7 for easy replacement. Upon sliding and/or snapping into place, the electrochemical sensing module 50 is adapted such that a tight seal is formed between the 15 inflow tubing 20 and the inflow orifice 54, and between the outflow orifice 56 and the outflow tubing 22.

20 Alternatively, the inflow and/or outflow tubing 20, 22 are part of the sensing module 50, and are also discarded and replaced after each use. The waste receptacle 16, collecting chamber, or the like, may also be part of the disposable sensing module 50. The electrical contacts for the IDA are also preferably adapted to plug-in to the controller 100 and/or a power supply once the module slides or snaps into place.

25 The sensor 80 may be any device that can detect and/or measure an electrochemical reporter that can undergo redox recycling, while providing for redox recycling of the electrochemical reporter. See, for example, WO 99/07879 and United States Patent No. 5,670,031. Preferred is an 30 interdigitated array of electrodes (IDA) with a spacing between the electrodes of about 800 μ m, or smaller. Most preferred is an array of electrodes with a spacing between

the electrodes between about 200 μ m and about 400 μ m, for example, an array of electrodes with a spacing between the electrodes of about 300 μ m.

5 The sensor 80 may have one or more IDAs. More IDAs provide for greater sensitivity, but are not necessarily indispensable, depending on the assay. When more than one array is present, (i.e., a "ganged" IDA sensor) the arrays may be linked in series or in parallel. Independent combinations thereof may also be used.

10 The sensor 80 is linked to a system controller 100 including a multipotentiostat that provides a specified potential across the IDA or IDAs and measures the dose dependent current resulting from redox recycling of electrochemical reporter molecules proximal to the IDA.

15 Alternatively, the information may be derived by scanning voltammetry, or the like. The system controller is thus capable of measuring and preferably also recording the change in voltage, and/or current, and the like, in the IDA. If more than one IDA is present in series, the system

20 controller 100 preferably can measure and also record the change occurring in each independent IDA or the sum of such changes. The system controller may advantageously be part of a computer network, such that processes and results can be order, monitored, controlled, retrieved and/or analyzed

25 remotely.

A magnetic field generating device 150, or the like, is positioned relative to the electrochemical sensing module 50 and is capable of generating a magnetic field of such strength that when a fluid having magnetic beads is

30 circulated within the chamber 52, a quantity of magnetic beads adequate for the detection or quantitation of the

analyte of interest will be attracted onto the sensor's 80 surface.

The magnetic field generating device 150 may be activated/deactivated by an on/off switch 152, or the like.

5 The switch may be under the control of a system controller 100, or the like.

Alternatively, the magnetic field generating device 150 may be a permanent magnet. In that case it is preferable that the magnet be moveable such that in at least a first 10 position the magnetic field it generates affects magnetic particles within chamber 52, such that it may cause magnetic beads to be attracted onto the sensor surface 80. When the magnet is moved into a second position the magnetic field does not significantly affect magnetic particles within the 15 chamber 52, such that the magnetic beads are no longer attracted onto the sensor surface 80, to facilitate clearing the magnetic beads from the sensor after the detection and/or quantitation of analyte has been achieved. An activatable/deactivatable magnetic field generating device 20 may be used, but is not necessarily required, when a single use/disposable electrochemical sensing module 50 is used.

In use, a buffer is pumped through the system and over the sensor 80 to establish a baseline. The buffer is flowed over the sensor 80 at any effective rate, however, a slow 25 rate (about 0.2mL/min) is preferred. Any effective buffer may be used, but enzyme substrate buffer (ESB), described below, is preferred.

The magnet 150 under the sensor 80 may be activated 152, or, alternatively, a magnet may be placed under the 30 sensor 80, at any time prior to the flow of the sample including the magnetic beads over the sensor 80. The magnet 150 should be able to generate an applied field such that an

adequate amount of magnetic beads may be drawn and captured on the surface of the sensor 80.

Next, the processed sample to be tested 12 is circulated over the sensor 80. The processed sample may be 5 prepared by any effective method. One method for preparing a processed sample is described in more detail *infra*. In general, the processed sample includes magnetic beads, or the like, and an enzyme indirectly linked to the magnetic beads by the analyte, the recognition molecule or the 10 recognition molecule/analyte complex.

The processed sample may be circulated over the sensor 80 surface for any effective amount of time, preferably until an adequate quantity of beads is captured by the magnet 150 over the sensor 80 surface. Preferably, the bead 15 solution is circulated for approximately 2 minutes at medium to fast flow rates (approximately 0.38mL/min). The effect of the magnet 150 and the flow rate should be such that an adequate concentration of beads is captured over the sensor surface 80.

20 A substrate is then circulated over the sensor 80. The substrate may be circulated at any effective rate, however, a slow flow rate (approximately 0.2mL/min) is preferred. The flow is then preferably stopped while substrate solution is over the sensor 80 and the signal is measured and/or 25 recorded by the system controller 100, with no flow, for the desired period of time.

The signal may be measured for any adequate amount of time. In general, however, the signal may be measured for about 90 to about 100 seconds or about 60 seconds of useable 30 data. Longer or shorter measurements may be used if necessary. It is within the skill in the art to determine

the optimal length of time the measurement should take place for a given set of conditions and samples.

5 The substrate will depend on the enzyme and the conditions used. Any effective substrate may be used. A non-exclusive list of enzyme/substrate pairs that may be used in accordance with the present invention is disclosed in WO 99/07879. Any effective concentration of substrate may be used. The preparation of the preferred substrate solution is described in greater detail *infra*.

10 Once the sample has been assayed, the beads may be cleared from the sensor 80 surface by deactivating 152, or removing, the magnet 150 from the proximity of the sensor 80, and circulating fresh buffer at a sufficiently rapid rate of flow over the sensor 80.

15 Alternatively, if a disposable sensor is being used, once the sample has been assayed the sensor module 50 may be removed and discarded.

20 In case the magnetic beads are to be cleared from the sensor, any effective buffer may be used, but ESB is preferred. The flow rate is preferably about 0.43mL/min. The addition of bubbles to the buffer flow has been found to assist the clearance of the beads. The washing buffer may be applied for any effective amount of time, however, generally between about 45 and 60 seconds has been found to 25 be sufficient. Once the beads are washed out, fresh buffer may be recycled over the sensor 80 until the baseline equilibrates again. This step generally takes about 30 seconds. The sensor 80 is then ready for a new sample.

30 Another aspect of the present invention is a fast and reliable assay for measuring and quantitating analytes in a sample. The method is particularly effective when used with the device of the present invention.

Analytes that may be detected or quantitated include any compound of interest for which there is a specific recognition molecule. Well known recognition molecules include proteins, such as receptors, immuno-globulins, and 5 the like; nucleic acids, their analogs, and the like; haptens; hormones; polypeptides; certain drugs; and other such molecules.

In general, the assay uses magnetic beads, or the like, which are commercially available. Any effective magnetic 10 beads may be used, however Tosyl-activated DYNABEADSM-450 (DYNAL Inc, 5 Delaware Drive, Lake Success, NY 11042 Prod No. 140.03, 140.04,) or the like, are preferred. The magnetic beads may be of any size that can be held to the chip surface with a magnetic field.

15 The magnetic beads are generally coated with a recognition molecule that binds with specificity and high affinity to the analyte to be detected or quantitated. Methods for coating magnetic beads with specific recognition molecules are well known in the art. The magnetic beads are 20 generally coated by dissolving the coating material in carbonate buffer (pH 9.6, 0.2 M) or the like, or any other well known in the art method.

For the DYNABEADS, the instructions provided by the manufacturer may be used. Briefly, the magnetic beads are 25 first resuspended and homogenized by vortexing, or the like, and a volume corresponding to the number of beads desired is pipetted into a test tube. The magnetic beads are concentrated using a magnet, and the supernatant is pipetted off, leaving the magnetic beads undisturbed.

30 The beads are then resuspended in an ample volume (preferably greater than original volume) of any effective buffer. It is within the skill in the art to determine the

most effective buffer for the recognition molecule to be used. Buffers that may be used include, for example, phosphate buffer pH 7.4, borate buffer pH 9.5 or acetate buffer pH 4.0 with molarities between 0.1M and 0.5M.

5 The beads are mixed gently with the final coating solution for any effective period of time. Generally, the beads are mixed with the final coating solution for about 2 minutes.

10 The magnetic beads are once more concentrated with a magnet, and the supernatant pipetted off leaving the beads undisturbed. The beads are then resuspended in an appropriate volume of any effective buffer. Effective buffers include, among other buffers, phosphate buffer pH 7.4, borate buffer pH 9.5 or acetate buffer pH 4.0. The 15 beads are now ready for coating.

For coating, the magnetic beads are thoroughly resuspended in any effective buffer. Effective buffers include, among other buffers, phosphate buffer pH 7.4, borate buffer pH 9.5 or acetate buffer pH 4.0. From between 20 about 1 μ g to about 10 μ g of the pure recognition molecule, if it is a protein, polypeptide or the like, per 10⁷ magnetic beads may be added to the magnetic bead/buffer solution. Preferably, about 5 μ g of the pure recognition molecule, if 25 it is a protein, polypeptide or the like, per 10⁷ magnetic beads is added to the magnetic bead/buffer solution. The solution is then vortexed for 1-2 minutes. The manufacturer of DYNABEADS recommends a concentration of 4-10 x 10⁸ DYNABEADS per ml final coating solution (including the antibody or other recognition molecule).

30 Preferably the salt concentration in the final coating solution is greater than about 0.05M. Higher pH and/or higher temperature will give a quicker formation of chemical

bonds. The upper pH and temperature limit is determined based on the recognition molecule used to coat the magnetic beads.

5 The magnetic beads/recognition molecule solution may then be incubated for 16-24 hours at 37°C with slow tilt rotation, or the like. Lower temperatures may be used for temperature sensitive recognition molecules. Higher temperatures and shorter incubation times may be used for stable recognition molecules. Preferably the magnetic beads 10 are not permitted to settle during the incubation period.

Phosphate buffer pH 7.4 (0.1M) may be prepared by dissolving 2.62 g $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ (MW 137.99) and 14.42 g $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ (MW 177.99) in distilled water and adjusting the volume to 1000 ml.

15 Borate buffer pH 9.5 (0.1M) may be prepared by dissolving 6.183 g H_3BO_3 (MW 61.83) in 800 ml distilled water, adjusting the pH to 9.5 using 5M NaOH and then adjusting the volume to 1000 ml with distilled water.

20 Acetate buffer pH 4.0 (0.1M) may be prepared by dissolving 2.86 ml acetic acid (CH_3COOH), in 900 ml distilled water, adjusting the pH to 4.0 using 5M NaOH and adjusting the volume to 1000 ml with distilled water.

25 These buffers may be used for prewashing and coating of DYNABEADSM-450 Tosylactivated. It is preferred that no proteins, sugars, or the like be added to these buffers.

Recognition molecules other than proteins or polypeptides may also be directly or indirectly used to coat the magnetic beads. For example, nucleic acids and their analogs can be attached to the magnetic beads by an avidin 30 biotin link, or the like; by binding the nucleic acid or analog to a protein like albumin or the like, which is then used to coat the magnetic beads; or by other methods well

known in the art. Other recognition molecules, including hormones, haptens, sugars, polypeptides and the like may similarly be bound to the magnetic beads using strategies well known by those of skill in the art.

5 After the incubation with the coating solution, the magnetic beads are concentrated using a magnet, and the supernatant is pipetted off. The coated beads are then washed, preferably a total of four times. Twice in buffer D for 5 minutes at 4°C, once in buffer E for 24 h at 20°C or
10 for 4 hours at 37°C, and once in buffer D for 5 minutes at 4°C. The beads should be coated and ready for use after this procedure. The amount of specific recognition molecules bound to the beads may be established by radioactive labeling, immunofluorescent methods,
15 spectrophotometry, or any other method known in the art.

The beads may be stored in buffer D at 4°C, usually for months, depending on the stability of the immobilized material. If the beads are stored for more than two weeks, it is preferred that they be washed twice in PBS/BSA for
20 five minutes before use.

Buffer D consists generally of PBS pH 7.4 with 0.1% w/v bovine serum albumin (BSA) or human serum albumin (HSA). It may be made by dissolving 0.88g NaCl (MW 58.4) and 0.1% (w/v) BSA or HSA to 80ml 0.01M Na-phosphate pH 7.4 (see
25 above). The solution is then mixed thoroughly and the volume adjusted to 100 ml with 0.01M Na-phosphate pH 7.4.

Buffer D is generally used for washing precoated DYNABEADS. According to the manufacturer, this buffer or any buffer containing protein or amino-groups (glycine, Tris
30 etc.) should preferably not be used for pre-washing or coating of DYNABEADSM-450 Tosylactivated.

If a preservative is needed in the coated product, an effective amount of sodium azide (NaN₃) may be added to buffer D. Preferred is a final concentration of 0.02% (w/v). This preservative is cytotoxic and should be 5 carefully removed before use by washing. Required safety precautions should be followed when handling this material.

Buffer E: 0.2M Tris pH 8.5 with 0.1% (w/v) BSA (HSA), may be made by dissolving 2.42g Tris in 80 ml distilled water, and adjusting the pH to 8.5 using 1 M HCl, then 10 dissolving 0.1% BSA/HSA and adjusting the volume to 100ml.

All reagents should preferably be analytical grade.

To test a sample for the presence or quantity of an analyte, the sample in which the analyte is to be detected or quantitated is combined with the coated beads in a 15 primary incubation. The primary incubation in general consists of adding the sample to be analyzed to the magnetic beads pre-coated with a first recognition molecule. In general, the volume in which the primary incubation is carried will depend on the number of beads to be used and 20 the final volume at which the reaction will take place.

Any effective number of beads per volume may be used in the primary incubation. The desired number of beads coated with the appropriate recognition molecule are pipetted and then washed in modified buffer E (MBE), which consists of 25 0.2 M Tris buffer, pH 8.5, with 1.0% (w/v) BSA, and are then resuspended in the desired volume of MBE. In general between about 4-5x10⁴ and about 4-5x10¹⁰ beads in 20μl may be used for an assay having a final volume of 40μl.

Preferably, between about 4-5x10⁵ and about 4-5x10⁷ beads in 30 20μl may be used for an assay having a final volume of 40μl. Most preferred is the use of between about 4-5x10⁶ and about

1×10^7 beads in 20 μ l for an assay having a final volume of 40 μ l.

Any sample generally tested using conventional techniques may also be tested using the methods and devices of the present invention. The sample may be diluted in MBE if necessary. In general, for example, it has been found that serum samples may be diluted 1:2 or 1:4, or even greater, if the analyte is present in sufficient concentrations. Diluting the sample has been found to decrease the background.

The sample and the beads are mixed, generally in a 1:1 (v/v) ratio. Preferably, 20 μ l of beads and 20 μ l of sample are mixed, for a total reaction volume of 40 μ l.

Several experiments have been performed in which the primary incubation time period was examined, with time incubation time intervals ranging from about 0.5 of a minute to about 30 minutes. Although longer incubations were found to yield more sensitive results, in general primary incubations of about 10 minutes or less were found to yield highly sensitive results. Primary incubations of about 5 minutes or less were also found to yield highly sensitive results. Most preferred are primary incubation of between about 1 and about 2 minutes.

After the primary incubation the beads are preferably washed twice with MBE (100 μ l per 40 μ l in the primary incubation may be used).

The secondary incubation with a conjugate, generally a second recognition molecule that specifically binds the analyte (or the first recognition molecule/analyte complex) and is conjugated or may be conjugated to an enzyme, is then effectuated. Other methods, for example, complementation of polypeptide fragments of beta-galactosidase, or the like,

may also be used. Any effective amount and concentration of the conjugate or second recognition molecule may be used. Preferably, however, the secondary incubation takes place in the same volume as the primary incubation. The conjugate 5 may be diluted in MBE, as necessary.

Secondary incubations ranging in time from about 0.5 of a minute to about 30 minutes were tried. Although longer incubations were found to yield more sensitive results, in general secondary incubations of about 10 minutes or less 10 were found to yield highly sensitive results. Secondary incubations of about 5 minutes or less were also found to yield highly sensitive results. Most preferred are secondary incubations of between about 1 and about 2 minutes. The solution is preferably gently rocked during 15 the procedure to ensure mixing of the reaction components.

It was found that when the systems and methods of the present invention are used, the primary and secondary incubations may be performed at room temperature (17°C - 25°C), with excellent results. Higher or lower temperatures 20 may be used if appropriate.

After the secondary incubation the liquid phase may be discarded and the reaction washed. In general, the reaction is washed three times in PBS with 0.05% TWEEN 20. Prior to injection into the device, the reaction is washed with 25 Enzyme substrate buffer (ESB), (0.1 M Phosphate, 0.1 M NaCl, pH 6.8), and the reaction resuspended in ESB. In general, with the device described above, the reaction is resuspended in 200µl.

The substrate to be used will depend on the enzyme in 30 the conjugate. In general, if the enzyme is beta-galactosidase, an effective substrate is P-aminophenyl-beta-D-galactopyranoside (PAPG). A concentration of 2mM is

preferred. A non-exclusive list of enzymes and substrates is disclosed in WO 99/07879.

Other assay formats known in the art may also be adapted for use in accordance with the present invention.

5 See, e.g., WO 99/07879.

Yet another embodiment of the present invention is a kit including reagents to perform the assays of the present invention. The kit may include any combination of reagents used in performing the assays. It may include, for example, 10 a first vial or the like having magnetic beads pre-coated with a recognition molecule for the analyte of interest; a second vial or the like having a second recognition molecule, specific for the analyte or the first recognition molecule/analyte complex, the second recognition molecule 15 being conjugated or conjugatable to an enzyme; a substrate, which in the presence of the enzyme generates an electrochemical capable of redox recycling. The kit may also contain a single use electrochemical sensor module. Preferably the kit also includes buffers, positive controls, 20 negative controls, and other reagents for use in the assay.

EXAMPLES

Experiments were conducted to evaluate faster, more sensitive devices and methods for detecting and quantitating analytes based on the proportional production of an 25 electrochemical capable of undergoing redox recycling and the measurement of the electrochemical with an IDA having a conformation such that the electrochemical will undergo redox recycling.

Unless otherwise specified, the following materials 30 were used. M450 Tosyl-activated magnetic beads (Dynal, Product No. 140.04). Coating buffer 0.1 M Phosphate Buffer Saline (PBS), pH 7.4. Post-coating washing buffer PBS, pH

7.4 with 0.1% (w/v) bovine serum albumin (BSA) (1x crystallized, Sigma, cat# A-4378). Storage buffer, PBS, pH 7.4 with 0.1% (w/v) BSA and 0.02% (w/v) sodium azide. Tosyl blocking buffer, 0.2 M Tris Buffer, pH 8.5, with 0.1% (w/v) BSA. Recombinant HIV-1 p24 antigen (Devaron, Inc., cat# 301-8-2, clone # AR-DEV). Human serum derived hepatitis B surface antigen AD subtype (adHBsAg) (Genzyme Diagnostics, Cat# ABH0707, Lot#M-22975). Recombinant HBsAg (ayw subtype) (Genzyme Diagnostics, Cat#ABH0705, Lot# M-22756). Goat 10 anti-human (IgG H+L-specific) conjugated to beta-galactosidase (American Qualex, cat# A110GN, lot# GG017). P-aminophenyl-beta-D-galactopyranoside (Sigma, cat# A-9545) at 2mM, in enzyme substrate buffer (0.1 M Phosphate, 0.1 M NaCl, pH 6.8).

15 Modified buffer E (MBE), 0.2 m Tris buffer, pH 8.5, with 1.0% (w/v) BSA, is used in the coating to block the unbound, active tosyl groups. It has been found that by using Tris buffer with BSA, the assay is less likely to produce non-specific binding.

20 The experiments were performed with the following positive and negative controls. Human serum with antibody to p24: negative α -p24 (98-058-08445), approximate titer of 0; low + α -p24 (98-053-01456), approximate titer of 261; medium + α -p24 (98-062-07940), approximate titer of 1,515; 25 and high + α -p24 (98-058-07537), approximate titer of 104,186. Human serum with antibody to HBsAg: high + α -HBsAg (98-306-04981), approximate concentration of 4742 mIU/ml; negative α -HBsAg (98-306-05415). Dilutions of high + α -HBsAg with the negative serum were used to produce samples 30 with lower α -HBsAg titers.

A colorimetric assay was performed on the samples for comparative purposes. This colorimetric assay is a widely utilized non-electrochemical detection technique. For the colorimetric bead optical endpoint assays, peroxidase-5 Affinipure F(ab) fragment mouse anti-human IgG Fc (gamma) fragment specific (Jackson Immunoresearch, code 209-036-098, lot 25206) was used and OPD was obtained from Abbott kit products (OPD tablets no. 7181E, OPD diluent no. 5695).

Example 1

10 The desired number of beads were washed in MBE and resuspended in the desired volume of MBE. In particular, 4-
5 $\times 10^6$ magnetic beads were used for an electrochemical reaction, while 1 $\times 10^6$ beads were used in the optical reaction.

15 The primary incubation in general consists of adding the sample to be analyzed to beads (20 μ l) pre-coated with the recognition molecule. For the test indicated below, the serum sample was diluted 1:4 in MBE (20 μ l per well) for a total reaction volume of 40 μ l.

20 Several experiments have been performed in which the primary incubation time period was examined, with time incubation time intervals ranging from about 0.5 of a minute to about 30 minutes. Although longer incubations were found to yield more sensitive results, in general primary 25 incubations of 1-2 minutes were found to yield a high sensitivity.

20 The reaction was then washed twice with MBE (100 μ l) and the beads incubated (secondary incubation) in goat-anti-human beta-galactosidase conjugate (40 μ l per well, 1:1000 dilution in MBE). Incubations ranging in time from about 0.5 of a minute to about 30 minutes were tried. Although longer secondary incubations were found to yield more

sensitive results. In general, however, incubations of 1-2 minutes were found to yield a high sensitivity. The solution is preferably shaken during the procedure to ensure mixing of the reaction components.

5 It was found that when the systems and methods of the present invention are used, the primary and secondary incubations may be performed at room temperature (17°C - 25°C), with excellent results.

10 After the secondary incubation, the liquid phase was discarded and the reaction washed three times (100µl) in PBS with 0.05% TWEEN 20 (PBST). The reaction was then washed once (100µl) with ESB, and the reaction resuspended in ESB (200µl).

15 The sensor (a single array of an interdigitated array of electrodes, as generally described in United States Patent No. 5,670,031) was activated and ESB flowed over the sensor at a slow rate (about 0.2mL/min) until a stable baseline was achieved. The magnet was then placed under the sensor. The magnet was placed such that it generated a 20 field of force sufficient to capture magnetic beads on the surface of the sensor.

25 The processed sample was then circulated over the sensor. The bead solution was circulated for approximately 2 minutes at medium to fast flow rates (approximately 0.38mL/min). Due to the magnet, a high concentration of beads was captured over the sensor surface.

30 The substrate (2mM PAPG, 100µl) was then circulated over the sensor at a slow flow rate (approximately 0.2mL/min). The flow was then stopped while substrate solution was over the sensor and the signal was measured with no flow for the desired period of time. The signal was

measured for about 90 to about 100 seconds for 60 seconds of useable data.

The beads were cleared from the sensor by removing the magnet from the proximity of the sensor and circulating 5 fresh ESB at a high flow rate (approximately 0.43mL/min) over the sensor. The addition of bubbles to the ESB flow was found to assist the clearance of the beads. The washing step generally took between about 45 and 60 seconds. Once 10 the beads were washed out, fresh ESB was recycled over the sensor until the baseline equilibrated. This step generally took about 30 seconds. The sensor was then ready for a new sample.

After coating the magnetic beads with p24, the beads were incubated for one minute with the serum to be tested 15 (primary incubation), washed, incubated for one minute with goat anti human beta-galactosidase IgG (secondary incubation) and washed. The procedure described above was then used to concentrate the beads over the sensor and the substrate was added.

20 Figure 2 is a graphical representation of the measured change in voltage over time. The first peak, starting at about t 11900 and ending at about t 12200 corresponds to the measurement of anti p24 in the low titer serum. The second peak, starting at about t 12500 and ending at about t 12800 25 corresponds to the measurement of anti p24 in the medium titer serum. The third peak, starting at about t 13200 and ending at about t 13500 corresponds to the measurement of anti p24 in the high titer serum.

The average slope was calculated from the data graph 30 (nAmp = y-axis; time (seconds) = x-axis) for data points acquired from the 20th second through the 100th second of measurement. Data was acquired at the rate of 2

observations per second and recorded as spreadsheet entries by the acquisition program (Origin Software). For the low titer serum the average slope was estimated to be 0.061, the average slope for the medium titer serum was estimated to be 5 0.112, and the average slope for the high titer serum was estimated to be 0.344. These values can be compared to the optical measurements obtained using a commercially available kit. The optical measurement provided values of 0.147, 0.291 and 0.495 for the low, medium and high titer serums 10 respectively. Advantageously, a tight correlation therefore was observed between the results obtained using the present invention and those obtained using commercially available systems. The results obtained using the present invention, however, required only a fraction of the time required for 15 the commercially available system and method.

Example 2

An experiment was conducted to find out the sensitivity of the systems and methods of the present invention under the conditions described below. Serial dilutions of human 20 serum having concentrations equivalent to 0, 15, 50, 100, 200, 400 and 800 mIU/ml anti-HBsAg were prepared. Magnetic beads (DYNABEADS M450) which had previously been coated with HBsAg were washed and resuspended in MBE.

For the primary incubation, the serum samples were 25 diluted 1:1 with MBE, and 25 μ l of each diluted sample was dispensed in a microtiter plate well. 5 \times 10⁶ coated beads in 25 μ l MBE were then added to each well. The samples were incubated for 2 minutes with gentle rocking at room temperature. The samples were then washed twice with MBE.

30 For the secondary incubation, 50 μ l of a 1:1000 dilution of goat anti human beta galactosidase conjugate in MBE was added to each well. The samples were incubated for 2

minutes with gentle rocking at room temperature. The samples were then washed twice with MBE, twice with PBST, once with ESB, and then resuspended in 250 μ l. The samples were then individually loaded onto the chip. PAPG 2mM in 5 ESB was then added to the system and the voltage in the sensor recorded.

Figure 3 is a graph plotting the slope of the kinetic measurement (nA/s) against the original concentration (mIU/ml) of HBsAg in the sample. The results indicate a 10 correlation having an R^2 equal to 0.8626. Qualitative results are obtainable for concentrations at least as low as 15 mIU/ml, with semi-quantitative results obtainable from 50 mIU/ml or greater under these conditions. As shown in Example 4 *infra*, more sensitive results may be obtained by 15 slightly varying the conditions.

Example 3

In this set of experiments, hepatitis B surface antigen (HBsAg) levels in human serum were measured. The measurement of dilutions corresponding to 0, 15, 50, 100, 200, 400 and 800mIU/ml were obtained using side by side 20 matched conditions for all reagents. A direct comparison was made between the sensitivity of the methods and devices in accordance with the present invention and the devices and methods disclosed in WO 99/07879, which are at least as 25 sensitive and reliable as the colorimetric assay commercially available, to obtain a direct comparison between the system and method with and without the novel aspects of the present invention.

Preliminarily, DYNABEADS (M450) (4×10^8) were coated with 30 200 μ g of HBsAg (100 μ g of ad subtype obtained from human plasma and 100 μ g of ayw recombinant HBsAg) in a 850 μ l reaction volume following the same protocol used for p24 in

Example 1. In parallel, a substantially identical surface area of a microtiter plate was also similarly coated.

Sets of microbeads and microtiter plate wells were then incubated (primary incubation) with the different dilutions 5 of HBsAg serum samples for two minutes at room temperature. After two minutes the samples were removed and the different sets of microbeads and the wells of the microtiter plates were washed. The microbeads and microtiter plate wells were then subjected to a two minutes secondary incubation with 10 goat anti-human β -galactosidase. The conjugate was then removed and excess conjugate was washed off.

The microbead samples corresponding to the different dilutions of the sample were then individually measured by capturing the microbeads over the sensor, adding the 15 substrate solution and measuring the change in voltage over a period of 60 seconds. Figure 4 is graphic representation of the results obtained.

Similarly, the matched pairs measured in the microtiter plates were assessed by stopping the reaction after two 20 minutes, and measuring in a traditional manner the electrochemical generated. Figure 5 is a graphic representation of the results obtained.

As can be seen by comparing figure 4 to figure 5, the method and device of the present invention provides under 25 these conditions a linear dose response that can qualitatively detect as low as 200mIU/ml anti-HBsAg at 2 standard deviations uncertainty, with a linear dose response up to 800 mIU/ml. In contrast, the results obtained using the traditional method showed no statistically significant 30 difference between the samples, i.e., the traditional method under these conditions does not demonstrate a measurable dose dependent increase in electrochemical, and in fact the

traditional method under these conditions cannot qualitatively detect the analyte at a concentration below 800 mIU/ml.

The sensitivity and reliability of the method and device, in particular as demonstrated by the results obtained using the short primary and secondary incubations at room temperature was much greater than expected. These properties of the methods and devices of the present invention are valuable because they permit faster, cheaper, 10 less cumbersome analysis of a sample.

Example 4

Having determined the unexpected and valuable properties of the system and method of the present invention, experiments were performed in order to optimize 15 the procedure. In this experiment, the effect of the concentration of magnetic beads per sample to be analyzed was evaluated.

The experiment was generally set up as in Example 2. Serum samples having three concentrations (100 mIU/ml, 500 20 mIU/ml, 2000 mIU/ml) of HBsAg were tested using either 800,000 beads per sample, as in Example 2, or 5,000,000 beads per serum sample. The data was derived as in Example 2, and is shown graphically in Figure 6.

The data from the experiment indicates that the 25 sensitivity of the device and method can be further greatly enhanced by increasing the number of magnetic beads per volume of sample. This presents a further advantage over the traditional method since it permits an increase in the surface area over which reactions can take place. As may be 30 seen from Figure 6, a concentration of 100 mIU/ml can easily be detected using the larger amount of beads. Lower concentrations were not tested, but the linearity of the

response indicates that concentrations as low as 15 mIU/ml should be easily obtained with the increased number of beads.

As shown in Fig. 7, another embodiment of the invention comprises forming linearly disposed discrete solution compartments within a conduit. Each solution compartment may be defined by interposing a separator, such as a gas bubble, within a carrier fluid at predetermined points. In this manner, the carrier fluid may be divided up into solution compartments, each of which is formed or sandwiched between two opposing gas bubbles within the conduit. The conduit, such as an inert tube, may be parallel to a ground surface, vertical to a ground surface, or even at an angle thereto. Preferably, the conduit is vertically positioned relative to a ground surface.

Each solution compartment may contain a different composition of materials, such as a sample or a conjugate, to respectively define a sample solution compartment or a conjugate solution compartment. At least one of the solutions compartments contains an attractable bead coated with a recognition molecule to define a coated bead solution compartment.

In operation, each of the solution compartments are transported over time, from left to right as seen in Fig. 7, within the conduit via a peristaltic pump or the like. An attraction device, such as a magnet, electromagnet, or the like, is disposed about the conduit. The attraction device, when actuated, is capable of attracting one or more of the attractable beads for processing/testing. The attraction device preferably contains a sensor, or IDA chip as described in detail *supra*. The sensor is capable of measuring the manipulated and processed beads after they

have been transported through the conduit and/or subjected to the "conveyor belt" of discrete solution compartments.

Advantageously, as each of the solution compartments are transported within the conduit, due to the placement of 5 the attraction device, the attraction device is capable of selectively retaining at least some of the attractable beads within the conduit. In this manner, the attracted attractable beads are effectively separated from the carrier fluid. As the carrier fluid continues to flow through the 10 conduit, the next linearly disposed solution compartment can manipulate the temporarily restrained beads. For example, if the next linear solution compartment comprises a wash solution, the temporarily restrained beads will be washed. Similarly, if the solution compartment preceding the wash 15 solution compartment contains a substrate/carrier fluid, the bathed and temporarily restrained beads can be subjected to the substrate/carrier fluid within the conduit.

As is apparent to one of ordinary skill in the art, such a conduit arrangement allows for the implementation of 20 separate processing steps in an endless sequence that can be manipulated depending on the assay. The preferred linear order of the solution compartments, as illustrated in Fig. 7 from left to right, is as follows: a substrate/carrier fluid compartment, a conjugate/carrier fluid compartment, a 25 sample/carrier fluid compartment, and a bead compartment. Most preferably, a wash solution compartment separates each of the four identified material-containing solution compartments. Multiple attraction devices are also preferably used to facilitate improved processing 30 techniques.

In sum, the preferred operational steps of this embodiment of the invention, as illustrated in Fig. 7, are

as follows: (1) transporting a coated bead solution compartment to a first separation station having an actuatable attraction device; (2) actuating the attraction device to attract some of the coated beads in the coated bead solution compartment such that some of the coated beads are temporarily restrained within the first separation station and separated from the carrier fluid; (3) flowing a wash solution compartment into the first separation station; (4) flowing a sample/carrier fluid solution compartment over the attracted beads; (5) actuating the attraction device to release the temporarily restrained beads into the sample solution compartment; (6) flowing the sample/bead mixture from the first separation station preferably to a second separation station having an actuatable attraction device; (7) actuating the second attraction device to attract some of the coated beads such that some of the coated beads are temporarily restrained within the second separation station; (8) flowing a wash solution compartment into the second separation station; (9) flowing a conjugate/carrier fluid solution compartment over the attracted beads; (10) actuating the second attraction device to release the temporarily restrained beads into the conjugate/carrier fluid solution compartment; (11) flowing the conjugate/bead mixture from the second separation station to a third separation station that preferably has a third actuatable attraction device having a sensor; (12) actuating the third actuatable attraction device to attract some of the beads, or more specifically, some of the bead/antigen/antibody/enzyme complex, to the vicinity of the sensor; (13) flowing a wash solution compartment into the third separation station; (14) flowing a substrate/carrier fluid solution compartment over the attracted beads, which

in the presence of the enzyme is cleaved into a reporter molecule capable of exhibiting redox recycling, and (15) measuring the presence or amount of electrochemical with the sensor, wherein the sensor produces redox recycling of the 5 electrochemical.

Thus, devices and methods for detecting and quantitating analytes in a sample are disclosed. While embodiments and applications of this invention have been shown and described, it will be apparent to those skilled in 10 the art that many modifications are possible without departing from the inventive concepts herein. The invention, therefore is not to be restricted except in the spirit of the appended claims.

We claim:

1. An electrochemical reporter device comprising:

- (a) a chamber for receiving an analytical reaction including magnetic beads;
- 5 (b) a sensor within the chamber, the sensor for detecting electrochemical reporter molecules within the chamber and the sensor having a configuration such that reporter molecules capable of exhibiting redox recycling will undergo redox recycling if within the chamber; and
- 10 (c) an actuatable magnetic field generating device selectively positioned such that magnetic beads present within the chamber will be attracted to the surface of the chamber wherein the sensor is located.

2. The electrochemical reporter device of claim 1, the sensor being a microelectronic interdigitated array of electrodes with a distance between the electrodes of about 20 100 to about 800 nanometers.

3. The electrochemical reporter device of claim 2, the sensor being a microelectronic interdigitated array of electrodes having a distance between the electrodes of about 25 300 nanometers.

4. An electrochemical reporter system comprising:

- (a) a magnetic bead;
- (b) a first recognition molecule capable of 30 specifically binding an analyte in a structure restricted manner, the recognition molecule being linked to the magnetic bead;

- (c) an enzyme;
- (d) a coupling element, for coupling with specificity the enzyme to the recognition molecule/analyte complex or the analyte;
- 5 (e) a substrate which in the presence of the enzyme is cleavable into a reporter molecule capable of exhibiting redox recycling;
- (f) a sensor for detecting the electrochemical reporter molecule, said sensor having a configuration such that the reporter molecule will exhibit redox recycling; and
- 10 (g) a magnetic field generating device positionable such that the magnetic beads may be attracted to the vicinity of the sensor.

15

5. The electrochemical reporter system of claim 4, the sensor being a microelectronic interdigitated array of electrodes with a distance between the electrodes of between about 100 to about 800 nanometers.

20

6. The electrochemical reporter system of claim 5, the distance between the electrodes being about 300 nanometers.

25

7. The electrochemical reporter system of claim 4, the enzyme being capable of effecting the cleavage of a covalent bond of the substrate.

30

8. The electrochemical reporter system of claim 7, the enzyme being selected from the group consisting of α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -mannosidase, β -mannosidase, acid phosphatase, alkaline phosphatase and phosphodiesterase II.

9. The electrochemical reporter system of claim 4, the substrate being selected from the group consisting of p-aminophenyl- β -D-galactopyranoside, p-aminophenyl- α -D-

5 galactopyranoside, p-aminophenyl- α -D-glucopyranoside, p-aminophenyl- β -D-glucopyranoside, p-aminophenyl- α -D-mannopyranoside, p-aminophenyl- β -D-mannopyranoside, p-aminophenylphosphate, and p-aminophenylphosphorylcholine.

10 10. The electrochemical reporter system of claim 4, the first recognition molecule being selected from the group consisting of a protein, a polypeptide, a nucleic acid, a nucleic acid analog, a hapten, immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, cell 15 adhesion molecules, receptors, non-biologic binding molecules and a hormone.

11. The electrochemical reporter system of claim 4, the coupling element comprising a second recognition molecule 20 coupled to an enzyme, the second recognition molecule being selected from the group consisting of a protein, a polypeptide, a nucleic acid, a nucleic acid analog, a hapten, immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, cell adhesion 25 molecules, receptors, non-biologic binding molecules and a hormone.

12. The electrochemical reporter system of claim 4, the substrate being cleaved into at least one component 30 comprising para-aminophenol.

13. The electrochemical reporter system of claim 4, the sensor being a microelectronic interdigitated array of electrodes having width between about 100 and about 800 nanometers and spaced between about 100 and about 800
5 nanometers from each other.

14. An assay for detecting or quantitating a specific analyte in a sample comprising the steps of:

- a) a primary incubation, wherein magnetic beads coated with a
10 first recognition molecule that specifically binds an analyte are incubated with a sample;
- b) a secondary incubation, wherein the magnetic beads are incubated with a conjugate comprising an enzyme and a second recognition molecule that specifically binds the
15 analyte, or the analyte/recognition molecule complex;
- c) capturing the magnetic beads with a magnet over a sensor capable of producing redox recycling of an electrochemical capable of undergoing redox recycling;
- d) adding a substrate, said substrate in the presence of the
20 enzyme being cleaved into an electrochemical capable of undergoing redox recycling; and
- e) detecting the presence or measuring the amount of electrochemical present in the solution with said sensor.

25 15. The assay of claim 14, the primary incubation lasting less than 10 minutes.

16. The assay of claim 14, the secondary incubation lasting less than 10 minutes.

30 17. The electrochemical reporter system of claim 14, the first recognition molecule being selected from the group

consisting of a protein, a polypeptide, a nucleic acid, a nucleic acid analog, a hapten, immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, cell adhesion molecules, receptors, non-biologic binding 5 molecules and a hormone.

18. The electrochemical reporter system of claim 14, the second recognition molecule being selected from the group consisting of a protein, a polypeptide, a nucleic acid, a nucleic acid analog, a hapten, immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, cell adhesion molecules, receptors, non-biologic binding 10 molecules and a hormone.

15 19. The electrochemical reporter system of claim 14, the enzyme being capable of effecting the cleavage of a covalent bond of the substrate.

20. The electrochemical reporter system of claim 19, the enzyme being selected from the group consisting of α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -mannosidase, β -mannosidase, acid phosphatase, alkaline phosphatase and phosphodiesterase II.

25 21. The electrochemical reporter system of claim 14, the substrate being selected from the group consisting of p-aminophenyl- β -D-galactopyranoside, p-aminophenyl- α -D-galactopyranoside, p-aminophenyl- α -D-glucopyranoside, p-aminophenyl- β -D-glucopyranoside, p-aminophenyl- α -D-30 mannoside, p-aminophenyl- β -D-mannopyranoside, p-aminophenylphosphate, and p-aminophenylphosphorylcholine.

22. The electrochemical reporter system of claim 14 wherein the substrate is cleaved into at least one component comprising para-aminophenol.

5 23. The electrochemical reporter system of claim 14 wherein the sensor is a microelectronic interdigitated array of electrodes having width between about 100 and about 800 nanometers and spaced between about 100 and about 800 nanometers from each other.

10

24. An electrochemical immunoassay for detecting an analyte in a sample comprising the steps of:

15 (a) having linked to a magnetic bead an antigen with an antibody specific for an analyte bound to the antigen, the antibody being coupled to an enzyme or having a coupling element for being specifically coupled to an enzyme;

20 (b) contacting the magnetic bead/antigen/antibody/enzyme complex with a sample to be analyzed;

25 (c) collecting the magnetic bead/antigen/antibody/enzyme complex;

(d) attracting the magnetic bead/antigen/antibody/enzyme complex to the vicinity of a sensor;

30 (e) adding a substrate to the collected magnetic bead/antigen/antibody/enzyme complex, the substrate in the presence of the enzyme being cleavable into a reporter molecule capable of exhibiting redox recycling; and

(f) measuring the presence or amount of reporter molecule with the sensor, the sensor being an

interdigitated array of electrodes capable of producing redox recycling of the reporter molecule.

5 25. An electrochemical assay for detecting a specific analyte in a sample comprising the steps of:

(a) having a recognition molecule linked to a magnetic bead, said recognition molecule capable of specifically binding the analyte in a structure

10 restricted manner;

(b) contacting the magnetic bead with a sample to be analyzed;

(c) coupling with specificity an enzyme to the recognition molecule or the analyte;

15 (d) attracting the magnetic bead/recognition molecule/analyte/enzyme conjugate complex to the vicinity of a sensor with a device capable of generating a magnetic field;

(e) adding a substrate, which in the presence of the enzyme is cleaved into a reporter molecule capable of exhibiting redox recycling; and

20 (f) measuring the presence or amount of electrochemical with the sensor, wherein the sensor is an interdigitated array of electrodes capable of producing redox recycling of the electrochemical.

25

26. A kit for detecting or measuring an analyte in a sample the kit comprising

30 i) magnetic beads pre-coated with a first recognition molecule specific for the analyte;

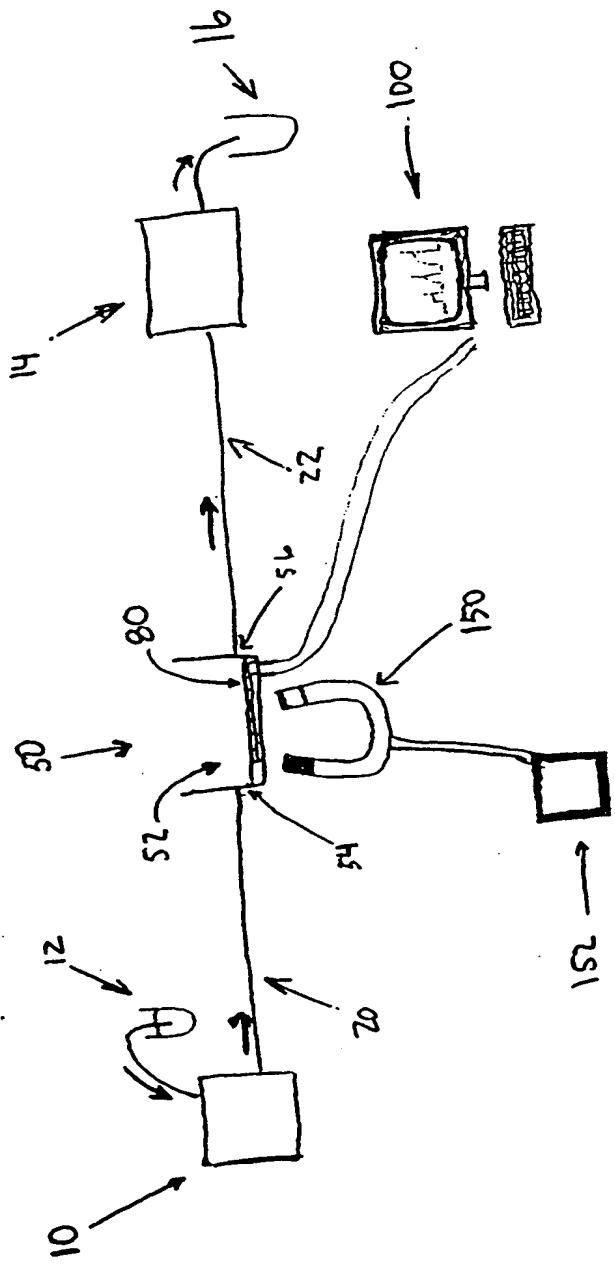
ii) a second recognition molecule, specific for the analyte or the first recognition molecule/analyte complex, the second recognition molecule being conjugated to an enzyme;
iii) a substrate which in the presence of the enzyme
5 generates an electrochemical capable of redox recycling.

27. The kit of claim 26 further comprising
iv) a single use electrochemical sensor module.

240/122

7

Fig. 1



L70/122

One minute incub. W/ serum
#One minute incub. W/ conjugate

P24 coated magnetic beads

Fig. 2

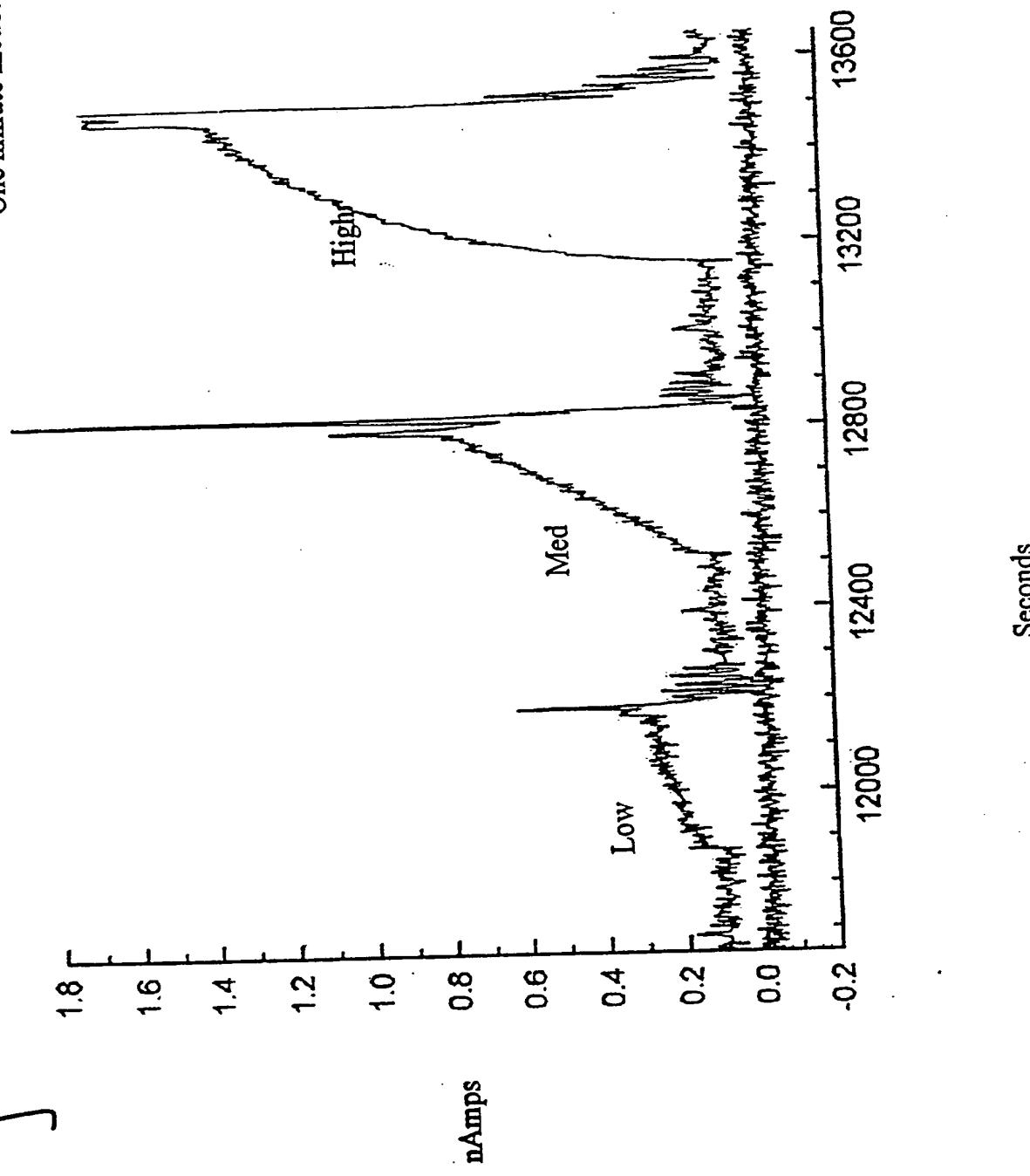


Fig. 3

Sample (mIU/mL)	+20 to +80 sec window	+20 s thru end of curve
	Slope (nA/sec)	Slope (nA/sec)
0	-0.0003	na
15	0.0002	0.0001
50	0.0002	0.0002
100	0.0005	0.0002
200	0.0008	0.0004
400	0.0011	0.0007
800	0.0016	0.0012

SENSITIVITY - TO 15 mIU/mL
FOR QUALITATIVE RESULT.

— TO 50 mIU/mL FOR
SEMI-QUANTITATIVE RESULT.

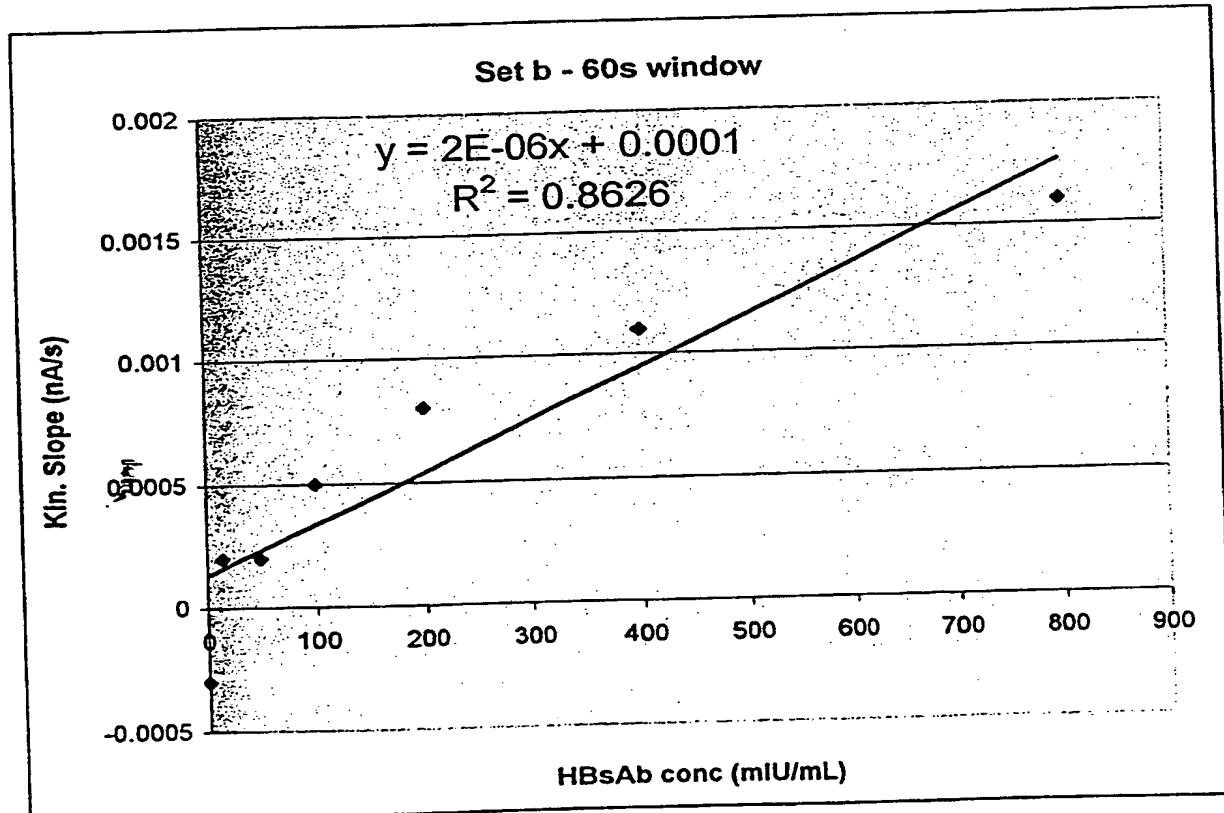


Fig. 4

std at 0.044
(2x max std)

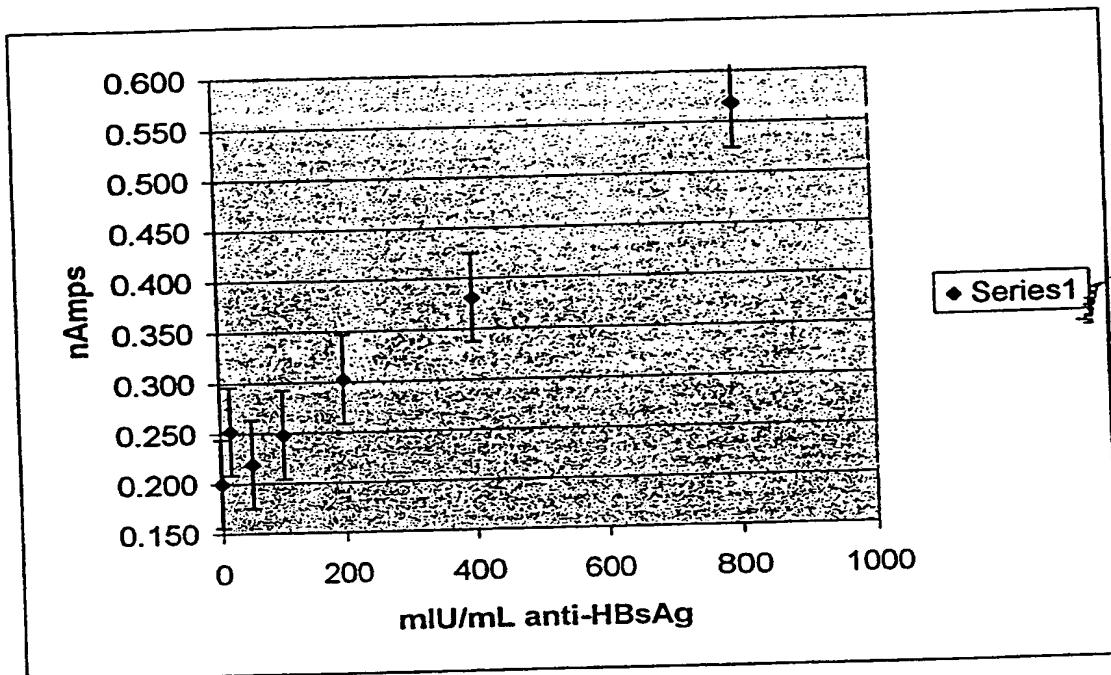


Fig. 5

STD | ERROR BARS @ 0.015

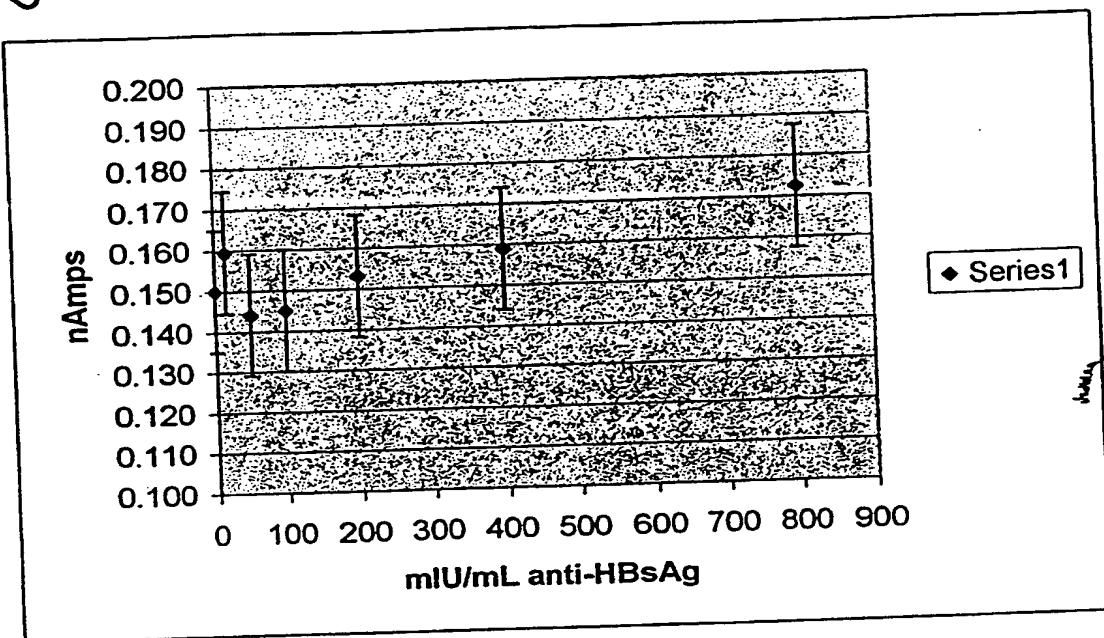
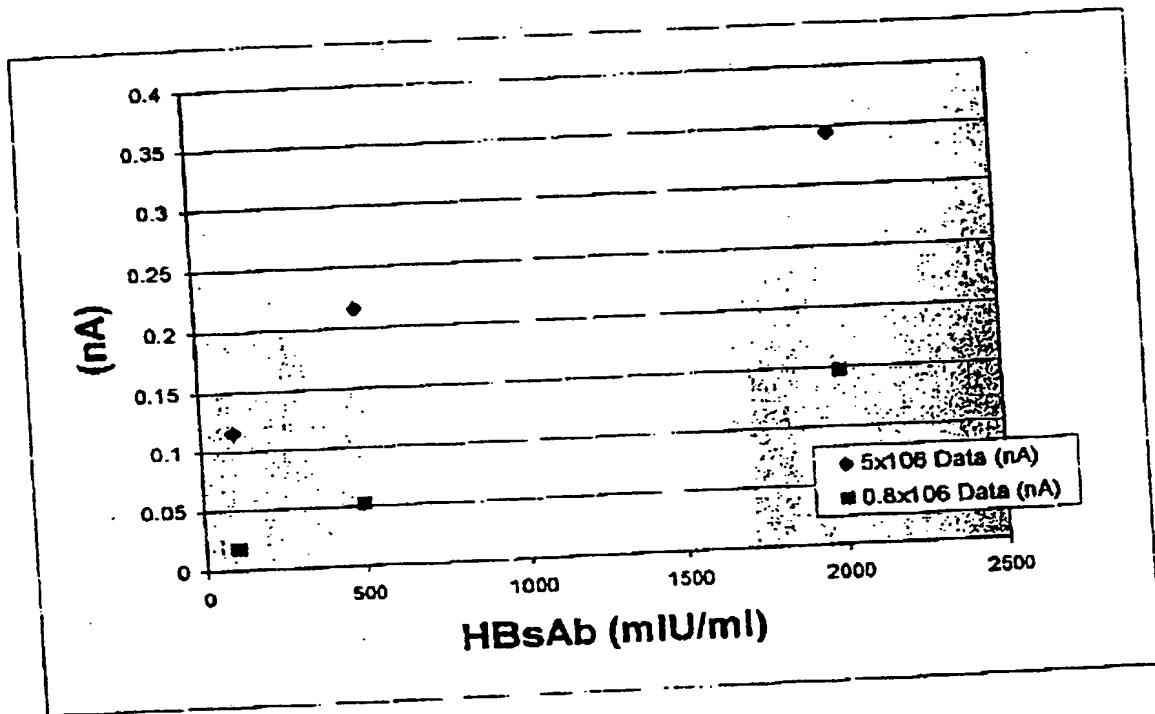
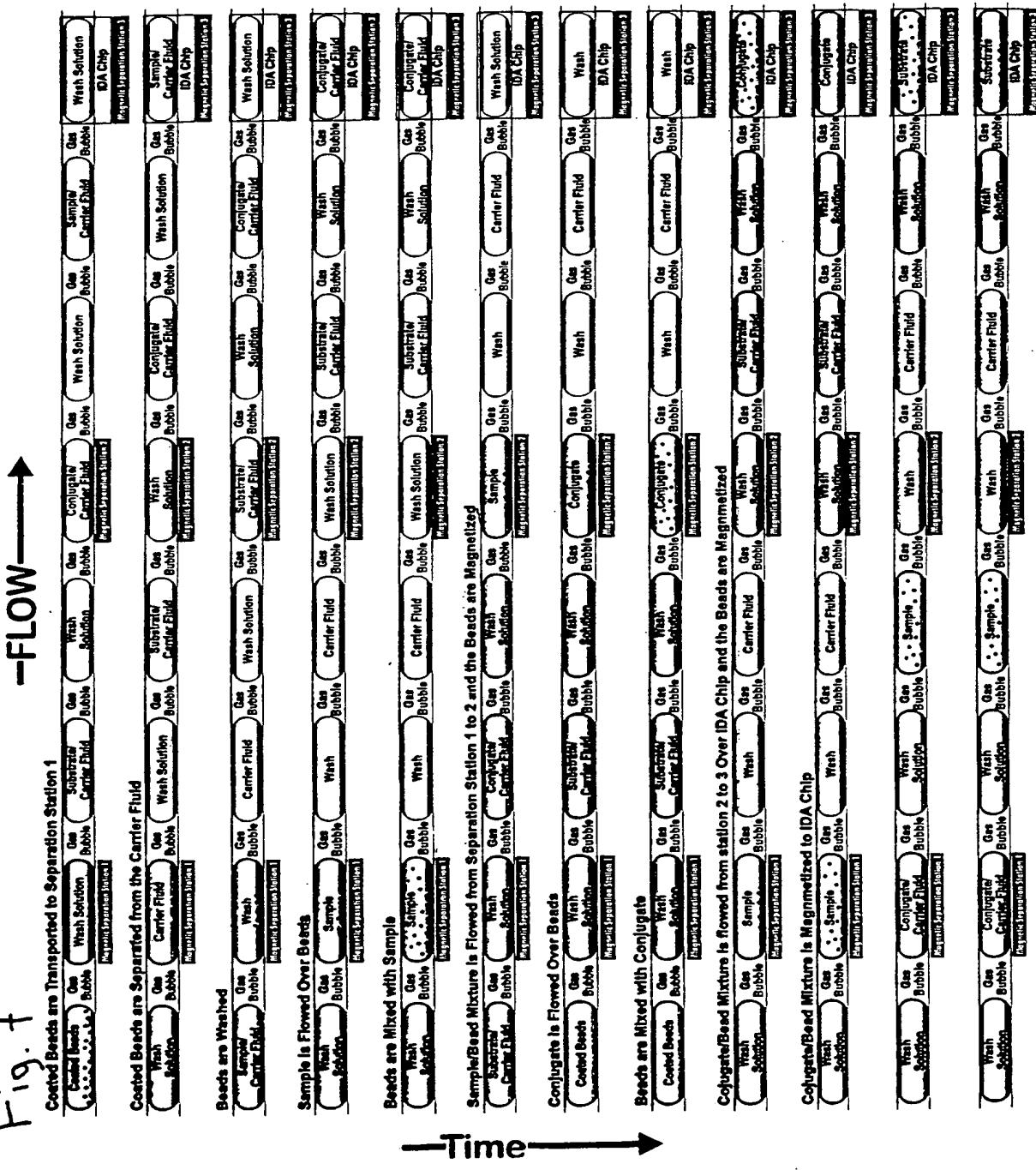


Fig. 6

HBsAb (mIU/ml)	5×10^5 Data (nA)	0.8×10^6 Data (nA)
2000	0.3439	0.1472
500	0.2158	0.052
100	0.116	0.0177



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二



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/03485

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N27/327 G01N33/543 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, COMPENDEX, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GEHRING A G ET AL: "Enzyme-linked immunomagnetic electrochemical detection of <i>Salmonella typhimurium</i> " JOURNAL OF IMMUNOLOGICAL METHODS, NL, ELSEVIER SCIENCE PUBLISHERS B.V., AMSTERDAM, vol. 195, no. 1, 9 September 1996 (1996-09-09), pages 15-25, XP004021249 ISSN: 0022-1759 page 16, column 1, paragraph 1 -page 16, column 2, paragraph 2 figure 2 ---	1,4, 7-12, 14-22, 26,27
Y	page 16, column 1, paragraph 1 -page 16, column 2, paragraph 2 figure 2 ---	24,25 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/03485

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	O NIWA ET AL: "Small-volume voltammetric detection of 4-aminophenol with interdigitated array electrodes and its application to electrochemical enzyme immunoassay" ANALYTICAL CHEMISTRY, US, AMERICAN CHEMICAL SOCIETY, COLUMBUS, vol. 65, no. 11, 1 January 1993 (1993-01-01), pages 1559-1563, XP002082750 ISSN: 0003-2700 abstract page 1560, column 1, paragraph 2 ---	24, 25
X	WEETALL H H ET AL: "A SIMPLE INEXPENSIVE DISPOSABLE ELECTROCHEMICAL SENSOR FOR CLINICAL AND IMMUNO-ASSAY" BIOSENSORS, vol. 3, no. 1, 1987, pages 57-64, XP000922945 ISSN: 0265-928X page 61, paragraph 2 -page 62, paragraph 1 figure 1 ---	1, 4, 7, 8, 10, 11, 14-20, 24-27
X	SANTANDREU M ET AL: "Development of electrochemical immunosensing systems with renewable surfaces" BIOSENSORS & BIOELECTRONICS, 1 JAN. 1998, ELSEVIER, UK, vol. 13, no. 1, pages 7-17, XP000922856 ISSN: 0956-5663 page 11 -page 12 figure 4 ---	1, 4, 7, 10, 11, 14-19, 26, 27
X	EP 0 859 229 A (GIST BROCADES BV) 19 August 1998 (1998-08-19) page 4, line 18 - line 28 page 4, line 49 - line 57 examples 4,5 ---	1, 4, 7, 8, 10, 11, 14-20, 26, 27
X	WO 86 05815 A (GENETICS INT INC) 9 October 1986 (1986-10-09) claim 21 -----	1, 4, 10, 11, 14-18, 26, 27

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Appl. No

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0859229	A 19-08-1998	AU 5301598	A	13-08-1998
WO 8605815	A 09-10-1986	AU 5667186	A	23-10-1986
		EP 0216844	A	08-04-1987

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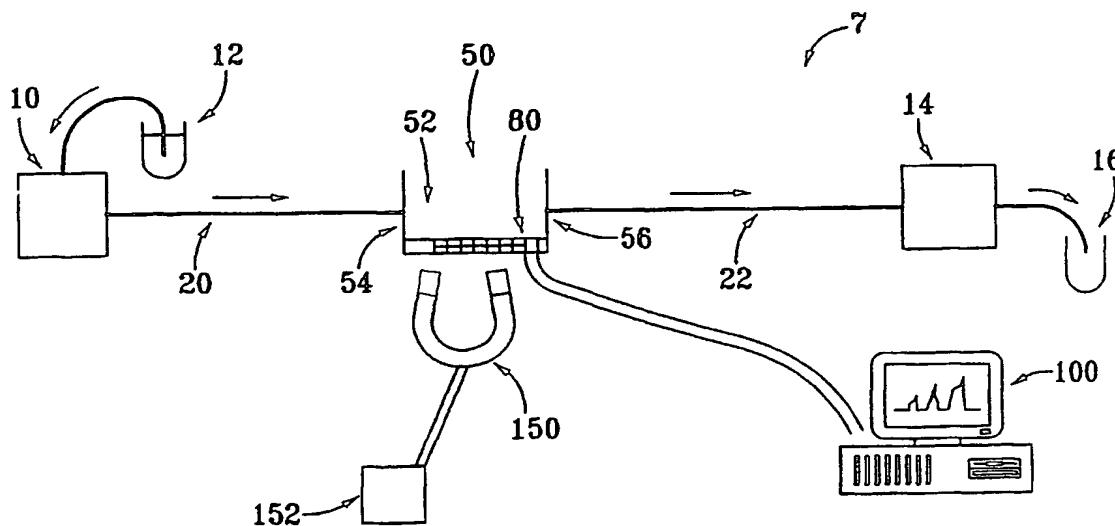
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(54) Title: ENZYME-LINKED IMMUNO-MAGNETIC ELECTROCHEMICAL BIOSENSOR



WO 00/47983 A1

(57) Abstract: A electrochemical biosensor system based on enzyme-linked immuno-magnetic sandwich assay wherein an interdigitated array of electrodes is equipped with a magnet to attract magnetic beads. Magnetic particles bear a first recognition molecule capable of binding to the analyte. Enzymes are chemically modified to complex with the analyte. When the sandwich assay is performed, a substrate is added. The substrate is chosen such that it is cleavable by the enzyme in a reporting molecule capable of redox recycling. A substrate when cleaved preferably leads to a p-aminophenol.



(15) Information about Correction:

see PCT Gazette No. 35/2001 of 30 August 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

DESCRIPTION

ENZYME-LINKED IMMUNO-MAGNETIC ELECTROCHEMICAL BIOSENSOR

FIELD OF THE INVENTION

The present invention relates to devices and methods for detecting and quantitating specific analytes in a sample.

5

BACKGROUND OF THE INVENTION

The detection and quantitation of specific analytes in a sample is an important activity in environmental, health, biotechnology, industrial chemistry and other fields. The 10 assays have also found use in high throughput screening, screening of oligo libraries in the field of functional genomics analysis, combinatorial chemistry screening, and other such fields. The analytes detected or quantitated may be any compound of interest for which there is a specific 15 recognition molecule. Well known recognition molecules include proteins, such as receptors, immuno-globulins, and the like, nucleic acids, their analogs, and the like, haptens, hormones, polypeptides, certain drugs and other such molecules.

20 Devices and techniques for detecting analytes are well known in the art. These including ELISAs, RIAs, PCR, and the like. Although these techniques have proven very powerful, effective and valuable, they suffer from drawbacks.

25 Most devices and techniques presently used for the detection of analytes require relatively long reaction

times, complex processes and laboratory conditions. For example, temperatures above room temperature, reaction times in excess of 30 minutes, and strict time limitations. Other drawbacks have included auto-fluorescence of reagents or analytes, in particular in the field of combinatorial chemistry, and when screening small peptide libraries using optical methods.

Decreasing the time necessary to perform an assay while maintaining the precision, sensitivity, reliability and dose-dependent results that can be obtained using conventional methods presents great economic advantages, and the patient's well-being in the case of laboratory medicine. The use of an electrochemical sensor rather than an optical sensor further presents other advantages, including avoiding auto-fluorescence and turbidity problems.

SUMMARY OF THE INVENTION

In a first, independent aspect of the present invention, an electrochemical sensor includes an interdigitated array of electrodes on a substantially dielectric substrate and a means for concentrating reagents on the surface of the interdigitated array of electrodes.

In a second, independent aspect of the present invention, an electrochemical reporter system includes a first recognition molecule linked to a magnetic bead, wherein the first recognition molecule can specifically bind an analyte; a second recognition molecule linked to an enzyme, for coupling with specificity the enzyme to the analyte or the first recognition molecule/analyte complex; a substrate, which in the presence of the enzyme is processed into an electrochemical reporter molecule capable of redox recycling; a sensor for detecting the electrochemical

reporter molecule, wherein the sensor has a configuration such that electrochemical reporter molecules, if present, exhibit redox recycling; and a magnetic field generating device positioned such that the magnetic field it generates 5 can attract to the surface of the sensor magnetic beads in solution over the sensor.

In a third, independent aspect of the present invention, an electrochemical reporter device includes a chamber for receiving an analytical reaction having magnetic 10 beads; a sensor on a surface of the chamber, the sensor for detecting electrochemical reporter molecules within the chamber, the sensor having a configuration such that it causes redox recycling of reporter molecules capable of exhibiting redox recycling; and a magnetic field generating 15 device capable of generating a magnetic field that attracts magnetic beads present within the chamber onto the sensor.

In a fourth, independent aspect of the present invention, an electrochemical reporter system includes a magnetic bead; a first recognition molecule capable of 20 specifically binding an analyte, the first recognition molecule being linked to the magnetic bead; an enzyme; a coupling element, or second recognition molecule, for coupling with specificity the enzyme to the analyte or the first recognition molecule/analyte complex; a substrate, 25 which in the presence of the enzyme is cleavable into an electrochemical reporter molecule capable of exhibiting redox recycling; a sensor for detecting the electrochemical reporter molecule and having a configuration such that the reporter molecule will exhibit redox recycling; and a 30 magnetic field generating device positioned such that the magnetic beads may be attracted to the vicinity of the sensor.

In a fifth, independent aspect of the present invention, an assay for detecting or quantitating a specific analyte in a sample comprises the following steps: a primary incubation, wherein magnetic beads coated with a recognition molecule that specifically binds an analyte are incubated with a sample; a secondary incubation, wherein the magnetic beads are then incubated with a conjugate comprising an enzyme and a molecule that specifically binds the analyte, or the analyte/recognition molecule complex; capturing the magnetic beads with a magnetic field generating device over a sensor capable of producing redox recycling of an electrochemical capable of undergoing redox recycling; adding a substrate, wherein the substrate in the presence of the enzyme is cleaved into an electrochemical reporter molecule capable of undergoing redox recycling; detecting the presence or measuring the amount of electrochemical present in the solution with the sensor.

In a sixth, independent aspect of the present invention, an electrochemical immunoassay for detecting an analyte in a sample includes the steps of providing an antigen linked to a magnetic bead and an antibody specific for an analyte bound to the antigen, wherein the antibody is coupled to an enzyme or has a coupling element such that it can be specifically coupled to an enzyme; contacting the magnetic bead/antigen/antibody/enzyme complex with a sample to be analyzed; attracting the magnetic bead/antigen/antibody/enzyme complex to the vicinity of a sensor; adding a substrate to the collected magnetic bead/antigen/antibody/enzyme complex, wherein the substrate in the presence of the enzyme is cleaved into an electrochemical reporter molecule capable of exhibiting redox recycling; detecting the presence or measuring the

amount of reporter molecule with the sensor, wherein the sensor is an interdigitated array of electrodes capable of producing redox recycling of the reporter molecule.

In a seventh, independent aspect of the present invention, an electrochemical immunoassay for detecting a specific analyte in a sample includes the steps of providing a recognition molecule linked to a magnetic bead, wherein the recognition molecule is capable of specifically binding the analyte; contacting the magnetic bead with a sample to be analyzed; coupling with specificity an enzyme to the analyte or the recognition molecule/analyte complex; attracting the magnetic bead/recognition molecule/analyte/coupling element-enzyme complex to the vicinity of a sensor with a device capable of generating a magnetic field; adding a substrate, which in the presence of the enzyme is cleaved into an electrochemical reporter molecule capable of exhibiting redox recycling; detecting the presence or measuring the amount of electrochemical with the sensor, wherein the sensor is an interdigitated array of electrodes capable of producing redox recycling of the electrochemical reporter molecule.

In an eighth, independent aspect of the present invention, an electrochemical reporter system includes a magnetic bead; a recognition molecule capable of specifically binding an analyte, the recognition molecule being linked to the magnetic bead; an enzyme; a coupling element, for coupling with specificity the enzyme to the analyte or recognition molecule/analyte complex; a substrate, which in the presence of the enzyme is cleavable into a reporter molecule capable of exhibiting redox recycling; a sensor, for detecting the electrochemical reporter molecule and having a configuration such that the

reporter molecule will exhibit redox recycling; a magnetic field generating device positioned such that the magnetic beads will be attracted to the vicinity of the sensor.

5 BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a schematic representation of a device in accordance with the present invention for a detecting and/or quantitating a specific analyte in a sample.

10 FIG. 2 is a graphic representation of the change in current measured when assaying in accordance with the present invention serum samples having low, medium and high anti-p24 levels.

15 FIG. 3 is a graph plotting the slope of the kinetic measurement (nA/s) against the original concentration (mIU/ml) of HBsAg in the sample.

FIG. 4 is a dose response curve using electrochemical measurement according to the present invention.

20 FIG. 5 are measurements of different concentrations using electrochemical measurement with a device without the magnetic beads and magnet of the present invention.

FIG. 6 is a comparison of assays using different number of magnetic beads per sample.

FIG. 7 is a flow chart of methods and devices in accordance with the present invention.

25

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Turning in detail to the drawings, FIG. 1 is a schematic representation of a device 7 in accordance with the present invention for detecting and/or quantitating a specific analyte in a sample.

A first pump 10 pumps a processed sample 12 through an inflow tubing, or first tubing segment 20, into the

electrochemical sensing module 50. The first pumping device 10 may be any device that can move fluids containing small particles, or a slurry, through a tubing segment. Preferably, the first pumping device 10 is an adjustable 5 speed peristaltic pump.

The first tubing segment 20 may be any tubing that can carry a fluid having small particles without clogging, or transport a fluid/particulate slurry. It is preferably made from an inert material, i.e., a material that will not 10 interact detrimentally with the fluids and reagents flowing within it. Most preferably, the first tubing segment 20 is TYGON tubing.

The diameter of the first tubing segment 20 will depend on the rate of flow desired. When the first pumping device 15 10 is a peristaltic pump, the rate of flow of fluids through the tube 20 can be increased by using tubing having a wider inner diameter, or by increasing the speed of the peristaltic pump. Conversely, a slower rate of flow can be achieved by using tubing having a smaller diameter or by 20 decreasing the speed of the peristaltic pump. Preferably the first tubing segment has an inner diameter such that an appropriate rate of flow may be achieved for the specific pump being used. The diameter of the tubing used will also be a function of the size of the beads and the fluid being 25 delivered.

The electrochemical sensing module 50, includes an inflow orifice 54, a chamber 52 for holding the sample, an outflow orifice 56 and a sensor 80. The outflow orifice 56 is connected to the outflow tubing, or second tubing segment 30 22. The second tubing segment 22 may be connected at its other end to a second pumping (drawing) device 14 (shown in the figure), or alternatively the first pumping device 10

may also be used for this purpose, in which case the system preferably is hermetically sealed.

The second tubing segment 22 advantageously terminates at a waste receptacle 16, collecting chamber, or the like.

5 The properties of the outflow tubing 22 are preferably the same as the inflow tubing 20, described *supra*. If two pumps are used, less flexible, more inert material can be used for the tubing, including TEFLON, or the like.

10 The electrochemical sensing module 50 may be a disposable, single use unit, in which case the module 50 preferably is adapted to slide and/or snap into and out of the device 7 for easy replacement. Upon sliding and/or snapping into place, the electrochemical sensing module 50 is adapted such that a tight seal is formed between the 15 inflow tubing 20 and the inflow orifice 54, and between the outflow orifice 56 and the outflow tubing 22.

20 Alternatively, the inflow and/or outflow tubing 20, 22 are part of the sensing module 50, and are also discarded and replaced after each use. The waste receptacle 16, collecting chamber, or the like, may also be part of the disposable sensing module 50. The electrical contacts for the IDA are also preferably adapted to plug-in to the controller 100 and/or a power supply once the module slides or snaps into place.

25 The sensor 80 may be any device that can detect and/or measure an electrochemical reporter that can undergo redox recycling, while providing for redox recycling of the electrochemical reporter. See, for example, WO 99/07879 and United States Patent No. 5,670,031. Preferred is an 30 interdigitated array of electrodes (IDA) with a spacing between the electrodes of about 800 μ m, or smaller. Most preferred is an array of electrodes with a spacing between

the electrodes between about 200 μ m and about 400 μ m, for example, an array of electrodes with a spacing between the electrodes of about 300 μ m.

5 The sensor 80 may have one or more IDAs. More IDAs provide for greater sensitivity, but are not necessarily indispensable, depending on the assay. When more than one array is present, (i.e., a "ganged" IDA sensor) the arrays may be linked in series or in parallel. Independent combinations thereof may also be used.

10 The sensor 80 is linked to a system controller 100 including a multipotentiostat that provides a specified potential across the IDA or IDAs and measures the dose dependent current resulting from redox recycling of electrochemical reporter molecules proximal to the IDA.

15 Alternatively, the information may be derived by scanning voltammetry, or the like. The system controller is thus capable of measuring and preferably also recording the change in voltage, and/or current, and the like, in the IDA. If more than one IDA is present in series, the system

20 controller 100 preferably can measure and also record the change occurring in each independent IDA or the sum of such changes. The system controller may advantageously be part of a computer network, such that processes and results can be ordered, monitored, controlled, retrieved and/or analyzed

25 remotely.

A magnetic field generating device 150, or the like, is positioned relative to the electrochemical sensing module 50 and is capable of generating a magnetic field of such strength that when a fluid having magnetic beads is 30 circulated within the chamber 52, a quantity of magnetic beads adequate for the detection or quantitation of the

analyte of interest will be attracted onto the sensor's 80 surface.

The magnetic field generating device 150 may be activated/deactivated by an on/off switch 152, or the like. 5 The switch may be under the control of a system controller 100, or the like.

Alternatively, the magnetic field generating device 150 may be a permanent magnet. In that case it is preferable that the magnet be moveable such that in at least a first 10 position the magnetic field it generates affects magnetic particles within chamber 52, such that it may cause magnetic beads to be attracted onto the sensor surface 80. When the magnet is moved into a second position the magnetic field does not significantly affect magnetic particles within the 15 chamber 52, such that the magnetic beads are no longer attracted onto the sensor surface 80, to facilitate clearing the magnetic beads from the sensor after the detection and/or quantitation of analyte has been achieved. An activatable/deactivatable magnetic field generating device 20 may be used, but is not necessarily required, when a single use/disposable electrochemical sensing module 50 is used.

In use, a buffer is pumped through the system and over the sensor 80 to establish a baseline. The buffer is flowed over the sensor 80 at any effective rate, however, a slow 25 rate (about 0.2mL/min) is preferred. Any effective buffer may be used, but enzyme substrate buffer (ESB), described below, is preferred.

The magnet 150 under the sensor 80 may be activated 152, or, alternatively, a magnet may be placed under the 30 sensor 80, at any time prior to the flow of the sample including the magnetic beads over the sensor 80. The magnet 150 should be able to generate an applied field such that an

adequate amount of magnetic beads may be drawn and captured on the surface of the sensor 80.

Next, the processed sample to be tested 12 is circulated over the sensor 80. The processed sample may be 5 prepared by any effective method. One method for preparing a processed sample is described in more detail *infra*. In general, the processed sample includes magnetic beads, or the like, and an enzyme indirectly linked to the magnetic beads by the analyte, the recognition molecule or the 10 recognition molecule/analyte complex.

The processed sample may be circulated over the sensor 80 surface for any effective amount of time, preferably until an adequate quantity of beads is captured by the magnet 150 over the sensor 80 surface. Preferably, the bead 15 solution is circulated for approximately 2 minutes at medium to fast flow rates (approximately 0.38mL/min). The effect of the magnet 150 and the flow rate should be such that an adequate concentration of beads is captured over the sensor surface 80.

20 A substrate is then circulated over the sensor 80. The substrate may be circulated at any effective rate, however, a slow flow rate (approximately 0.2mL/min) is preferred. The flow is then preferably stopped while substrate solution is over the sensor 80 and the signal is measured and/or 25 recorded by the system controller 100, with no flow, for the desired period of time.

The signal may be measured for any adequate amount of time. In general, however, the signal may be measured for about 90 to about 100 seconds or about 60 seconds of useable 30 data. Longer or shorter measurements may be used if necessary. It is within the skill in the art to determine

the optimal length of time the measurement should take place for a given set of conditions and samples.

The substrate will depend on the enzyme and the conditions used. Any effective substrate may be used. A 5 non-exclusive list of enzyme/substrate pairs that may be used in accordance with the present invention is disclosed in WO 99/07879. Any effective concentration of substrate may be used. The preparation of the preferred substrate solution is described in greater detail *infra*.

10 Once the sample has been assayed, the beads may be cleared from the sensor 80 surface by deactivating 152, or removing, the magnet 150 from the proximity of the sensor 80, and circulating fresh buffer at a sufficiently rapid rate of flow over the sensor 80.

15 Alternatively, if a disposable sensor is being used, once the sample has been assayed the sensor module 50 may be removed and discarded.

20 In case the magnetic beads are to be cleared from the sensor, any effective buffer may be used, but ESB is preferred. The flow rate is preferably about 0.43mL/min. The addition of bubbles to the buffer flow has been found to assist the clearance of the beads. The washing buffer may be applied for any effective amount of time, however, generally between about 45 and 60 seconds has been found to 25 be sufficient. Once the beads are washed out, fresh buffer may be recycled over the sensor 80 until the baseline equilibrates again. This step generally takes about 30 seconds. The sensor 80 is then ready for a new sample.

30 Another aspect of the present invention is a fast and reliable assay for measuring and quantitating analytes in a sample. The method is particularly effective when used with the device of the present invention.

Analytes that may be detected or quantitated include any compound of interest for which there is a specific recognition molecule. Well known recognition molecules include proteins, such as receptors, immuno-globulins, and 5 the like; nucleic acids, their analogs, and the like; haptens; hormones; polypeptides; certain drugs; and other such molecules.

In general, the assay uses magnetic beads, or the like, which are commercially available. Any effective magnetic 10 beads may be used, however Tosyl-activated DYNABEADSM-450 (DYNAL Inc, 5 Delaware Drive, Lake Success, NY 11042 Prod No. 140.03, 140.04,) or the like, are preferred. The magnetic beads may be of any size that can be held to the chip surface with a magnetic field.

15 The magnetic beads are generally coated with a recognition molecule that binds with specificity and high affinity to the analyte to be detected or quantitated. Methods for coating magnetic beads with specific recognition molecules are well known in the art. The magnetic beads are 20 generally coated by dissolving the coating material in carbonate buffer (pH 9.6, 0.2 M) or the like, or any other well known in the art method.

For the DYNABEADS, the instructions provided by the manufacturer may be used. Briefly, the magnetic beads are 25 first resuspended and homogenized by vortexing, or the like, and a volume corresponding to the number of beads desired is pipetted into a test tube. The magnetic beads are concentrated using a magnet, and the supernatant is pipetted off, leaving the magnetic beads undisturbed.

30 The beads are then resuspended in an ample volume (preferably greater than original volume) of any effective buffer. It is within the skill in the art to determine the

most effective buffer for the recognition molecule to be used. Buffers that may be used include, for example, phosphate buffer pH 7.4, borate buffer pH 9.5 or acetate buffer pH 4.0 with molarities between 0.1M and 0.5M.

5 The beads are mixed gently with the final coating solution for any effective period of time. Generally, the beads are mixed with the final coating solution for about 2 minutes.

10 The magnetic beads are once more concentrated with a magnet, and the supernatant pipetted off leaving the beads undisturbed. The beads are then resuspended in an appropriate volume of any effective buffer. Effective buffers include, among other buffers, phosphate buffer pH 7.4, borate buffer pH 9.5 or acetate buffer pH 4.0. The 15 beads are now ready for coating.

For coating, the magnetic beads are thoroughly resuspended in any effective buffer. Effective buffers include, among other buffers, phosphate buffer pH 7.4, borate buffer pH 9.5 or acetate buffer pH 4.0. From between 20 about 1 μ g to about 10 μ g of the pure recognition molecule, if it is a protein, polypeptide or the like, per 10⁷ magnetic beads may be added to the magnetic bead/buffer solution. Preferably, about 5 μ g of the pure recognition molecule, if 25 it is a protein, polypeptide or the like, per 10⁷ magnetic beads is added to the magnetic bead/buffer solution. The solution is then vortexed for 1-2 minutes. The manufacturer of DYNABEADS recommends a concentration of 4-10 x 10⁸ DYNABEADS per ml final coating solution (including the antibody or other recognition molecule).

30 Preferably the salt concentration in the final coating solution is greater than about 0.05M. Higher pH and/or higher temperature will give a quicker formation of chemical

bonds. The upper pH and temperature limit is determined based on the recognition molecule used to coat the magnetic beads.

The magnetic beads/recognition molecule solution may 5 then be incubated for 16-24 hours at 37°C with slow tilt rotation, or the like. Lower temperatures may be used for temperature sensitive recognition molecules. Higher temperatures and shorter incubation times may be used for stable recognition molecules. Preferably the magnetic beads 10 are not permitted to settle during the incubation period.

Phosphate buffer pH 7.4 (0.1M) may be prepared by dissolving 2.62 g $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ (MW 137.99) and 14.42 g $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ (MW 177.99) in distilled water and adjusting the volume to 1000 ml.

15 Borate buffer pH 9.5 (0.1M) may be prepared by dissolving 6.183 g H_3BO_3 (MW 61.83) in 800 ml distilled water, adjusting the pH to 9.5 using 5M NaOH and then adjusting the volume to 1000 ml with distilled water.

20 Acetate buffer pH 4.0 (0.1M) may be prepared by dissolving 2.86 ml acetic acid (CH_3COOH), in 900 ml distilled water, adjusting the pH to 4.0 using 5M NaOH and adjusting the volume to 1000 ml with distilled water.

These buffers may be used for prewashing and coating of 25 DYNABEADSM-450 Tosylactivated. It is preferred that no proteins, sugars, or the like be added to these buffers.

Recognition molecules other than proteins or 30 polypeptides may also be directly or indirectly used to coat the magnetic beads. For example, nucleic acids and their analogs can be attached to the magnetic beads by an avidin biotin link, or the like; by binding the nucleic acid or analog to a protein like albumin or the like, which is then used to coat the magnetic beads; or by other methods well

known in the art. Other recognition molecules, including hormones, haptens, sugars, polypeptides and the like may similarly be bound to the magnetic beads using strategies well known by those of skill in the art.

5 After the incubation with the coating solution, the magnetic beads are concentrated using a magnet, and the supernatant is pipetted off. The coated beads are then washed, preferably a total of four times. Twice in buffer D for 5 minutes at 4°C, once in buffer E for 24 h at 20°C or
10 for 4 hours at 37°C, and once in buffer D for 5 minutes at 4°C. The beads should be coated and ready for use after this procedure. The amount of specific recognition molecules bound to the beads may be established by radioactive labeling, immunofluorescent methods,
15 spectrophotometry, or any other method known in the art.

The beads may be stored in buffer D at 4°C, usually for months, depending on the stability of the immobilized material. If the beads are stored for more than two weeks, it is preferred that they be washed twice in PBS/BSA for
20 five minutes before use.

Buffer D consists generally of PBS pH 7.4 with 0.1% w/v bovine serum albumin (BSA) or human serum albumin (HSA). It may be made by dissolving 0.88g NaCl (MW 58.4) and 0.1% (w/v) BSA or HSA to 80ml 0.01M Na-phosphate pH 7.4 (see
25 above). The solution is then mixed thoroughly and the volume adjusted to 100 ml with 0.01M Na-phosphate pH 7.4.

Buffer D is generally used for washing precoated DYNABEADS. According to the manufacturer, this buffer or any buffer containing protein or amino-groups (glycine, Tris
30 etc.) should preferably not be used for pre-washing or coating of DYNABEADSM-450 Tosylactivated.

If a preservative is needed in the coated product, an effective amount of sodium azide (NaN₃) may be added to buffer D. Preferred is a final concentration of 0.02% (w/v). This preservative is cytotoxic and should be 5 carefully removed before use by washing. Required safety precautions should be followed when handling this material.

Buffer E: 0.2M Tris pH 8.5 with 0.1% (w/v) BSA (HSA), may be made by dissolving 2.42g Tris in 80 ml distilled water, and adjusting the pH to 8.5 using 1 M HCl, then 10 dissolving 0.1% BSA/HSA and adjusting the volume to 100ml.

All reagents should preferably be analytical grade.

To test a sample for the presence or quantity of an analyte, the sample in which the analyte is to be detected or quantitated is combined with the coated beads in a 15 primary incubation. The primary incubation in general consists of adding the sample to be analyzed to the magnetic beads pre-coated with a first recognition molecule. In general, the volume in which the primary incubation is carried will depend on the number of beads to be used and 20 the final volume at which the reaction will take place.

Any effective number of beads per volume may be used in the primary incubation. The desired number of beads coated with the appropriate recognition molecule are pipetted and then washed in modified buffer E (MBE), which consists of 25 0.2 m Tris buffer, pH 8.5, with 1.0% (w/v) BSA, and are then resuspended in the desired volume of MBE. In general between about 4-5x10⁴ and about 4-5x10¹⁰ beads in 20μl may be used for an assay having a final volume of 40μl.

Preferably, between about 4-5x10⁵ and about 4-5x10⁷ beads in 30 20μl may be used for an assay having a final volume of 40μl. Most preferred is the use of between about 4-5x10⁶ and about

1×10^7 beads in 20 μ l for an assay having a final volume of 40 μ l.

Any sample generally tested using conventional techniques may also be tested using the methods and devices 5 of the present invention. The sample may be diluted in MBE if necessary. In general, for example, it has been found that serum samples may be diluted 1:2 or 1:4, or even greater, if the analyte is present in sufficient concentrations. Diluting the sample has been found to 10 decrease the background.

The sample and the beads are mixed, generally in a 1:1 (v/v) ratio. Preferably, 20 μ l of beads and 20 μ l of sample are mixed, for a total reaction volume of 40 μ l.

Several experiments have been performed in which the 15 primary incubation time period was examined, with time incubation time intervals ranging from about 0.5 of a minute to about 30 minutes. Although longer incubations were found to yield more sensitive results, in general primary incubations of about 10 minutes or less were found to yield 20 highly sensitive results. Primary incubations of about 5 minutes or less were also found to yield highly sensitive results. Most preferred are primary incubation of between about 1 and about 2 minutes.

After the primary incubation the beads are preferably 25 washed twice with MBE (100 μ l per 40 μ l in the primary incubation may be used).

The secondary incubation with a conjugate, generally a second recognition molecule that specifically binds the analyte (or the first recognition molecule/analyte complex) 30 and is conjugated or may be conjugated to an enzyme, is then effectuated. Other methods, for example, complementation of polypeptide fragments of beta-galactosidase, or the like,

may also be used. Any effective amount and concentration of the conjugate or second recognition molecule may be used. Preferably, however, the secondary incubation takes place in the same volume as the primary incubation. The conjugate 5 may be diluted in MBE, as necessary.

Secondary incubations ranging in time from about 0.5 of a minute to about 30 minutes were tried. Although longer incubations were found to yield more sensitive results, in general secondary incubations of about 10 minutes or less 10 were found to yield highly sensitive results. Secondary incubations of about 5 minutes or less were also found to yield highly sensitive results. Most preferred are secondary incubations of between about 1 and about 2 minutes. The solution is preferably gently rocked during 15 the procedure to ensure mixing of the reaction components.

It was found that when the systems and methods of the present invention are used, the primary and secondary incubations may be performed at room temperature (17°C - 25°C), with excellent results. Higher or lower temperatures 20 may be used if appropriate.

After the secondary incubation the liquid phase may be discarded and the reaction washed. In general, the reaction is washed three times in PBS with 0.05% TWEEN 20. Prior to injection into the device, the reaction is washed with 25 Enzyme substrate buffer (ESB), (0.1 M Phosphate, 0.1 M NaCl, pH 6.8), and the reaction resuspended in ESB. In general, with the device described above, the reaction is resuspended in 200µl.

The substrate to be used will depend on the enzyme in 30 the conjugate. In general, if the enzyme is beta-galactosidase, an effective substrate is P-aminophenyl-beta-D-galactopyranoside (PAPG). A concentration of 2mM is

preferred. A non-exclusive list of enzymes and substrates is disclosed in WO 99/07879.

Other assay formats known in the art may also be adapted for use in accordance with the present invention.

5 See, e.g., WO 99/07879.

Yet another embodiment of the present invention is a kit including reagents to perform the assays of the present invention. The kit may include any combination of reagents used in performing the assays. It may include, for example, 10 a first vial or the like having magnetic beads pre-coated with a recognition molecule for the analyte of interest; a second vial or the like having a second recognition molecule, specific for the analyte or the first recognition molecule/analyte complex, the second recognition molecule 15 being conjugated or conjugatable to an enzyme; a substrate, which in the presence of the enzyme generates an electrochemical capable of redox recycling. The kit may also contain a single use electrochemical sensor module. Preferably the kit also includes buffers, positive controls, 20 negative controls, and other reagents for use in the assay.

EXAMPLES

Experiments were conducted to evaluate faster, more sensitive devices and methods for detecting and quantitating analytes based on the proportional production of an 25 electrochemical capable of undergoing redox recycling and the measurement of the electrochemical with an IDA having a conformation such that the electrochemical will undergo redox recycling.

Unless otherwise specified, the following materials 30 were used. M450 Tosyl-activated magnetic beads (Dynal, Product No. 140.04). Coating buffer 0.1 M Phosphate Buffer Saline (PBS), pH 7.4. Post-coating washing buffer PBS, pH

7.4 with 0.1% (w/v) bovine serum albumin (BSA) (1x crystallized, Sigma, cat# A-4378). Storage buffer, PBS, pH 7.4 with 0.1% (w/v) BSA and 0.02% (w/v) sodium azide. Tosyl blocking buffer, 0.2 M Tris Buffer, pH 8.5, with 0.1% (w/v) BSA. Recombinant HIV-1 p24 antigen (Devaron, Inc., cat# 301-8-2, clone # AR-DEV). Human serum derived hepatitis B surface antigen AD subtype (adHBsAg) (Genzyme Diagnostics, Cat# ABH0707, Lot#M-22975). Recombinant HBsAg (ayw subtype) (Genzyme Diagnostics, Cat#ABH0705, Lot# M-22756). Goat 10 anti-human (IgG H+L-specific) conjugated to beta-galactosidase (American Qualex, cat# A110GN, lot# GG017). P-aminophenyl-beta-D-galactopyranoside (Sigma, cat# A-9545) at 2mM, in enzyme substrate buffer (0.1 M Phosphate, 0.1 M NaCl, pH 6.8).

15 Modified buffer E (MBE), 0.2 m Tris buffer, pH 8.5, with 1.0% (w/v) BSA, is used in the coating to block the unbound, active tosyl groups. It has been found that by using Tris buffer with BSA, the assay is less likely to produce non-specific binding.

20 The experiments were performed with the following positive and negative controls. Human serum with antibody to p24: negative α -p24 (98-058-08445), approximate titer of 0; low + α -p24 (98-053-01456), approximate titer of 261; medium + α -p24 (98-062-07940), approximate titer of 1,515; 25 and high + α -p24 (98-058-07537), approximate titer of 104,186. Human serum with antibody to HBsAg: high + α -HBsAg (98-306-04981), approximate concentration of 4742 mIU/ml; negative α -HBsAg (98-306-05415). Dilutions of high + α -HBsAg with the negative serum were used to produce samples 30 with lower α -HBsAg titers.

A colorimetric assay was performed on the samples for comparative purposes. This colorimetric assay is a widely utilized non-electrochemical detection technique. For the colorimetric bead optical endpoint assays, peroxidase-5 Affinipure F(ab) fragment mouse anti-human IgG Fc (gamma) fragment specific (Jackson Immunoresearch, code 209-036-098, lot 25206) was used and OPD was obtained from Abbott kit products (OPD tablets no. 7181E, OPD diluent no. 5695).

Example 1

10 The desired number of beads were washed in MBE and resuspended in the desired volume of MBE. In particular, 4-
5 \times 10⁶ magnetic beads were used for an electrochemical reaction, while 1 \times 10⁶ beads were used in the optical reaction.

15 The primary incubation in general consists of adding the sample to be analyzed to beads (20 μ l) pre-coated with the recognition molecule. For the test indicated below, the serum sample was diluted 1:4 in MBE (20 μ l per well) for a total reaction volume of 40 μ l.

20 Several experiments have been performed in which the primary incubation time period was examined, with time incubation time intervals ranging from about 0.5 of a minute to about 30 minutes. Although longer incubations were found to yield more sensitive results, in general primary 25 incubations of 1-2 minutes were found to yield a high sensitivity.

The reaction was then washed twice with MBE (100 μ l) and the beads incubated (secondary incubation) in goat-anti-30 human beta-galactosidase conjugate (40 μ l per well, 1:1000 dilution in MBE). Incubations ranging in time from about 0.5 of a minute to about 30 minutes were tried. Although longer secondary incubations were found to yield more

sensitive results. In general, however, incubations of 1-2 minutes were found to yield a high sensitivity. The solution is preferably shaken during the procedure to ensure mixing of the reaction components.

5 It was found that when the systems and methods of the present invention are used, the primary and secondary incubations may be performed at room temperature (17°C - 25°C), with excellent results.

10 After the secondary incubation, the liquid phase was discarded and the reaction washed three times (100µl) in PBS with 0.05% TWEEN 20 (PBST). The reaction was then washed once (100µl) with ESB, and the reaction resuspended in ESB (200µl).

15 The sensor (a single array of an interdigitated array of electrodes, as generally described in United States Patent No. 5,670,031) was activated and ESB flowed over the sensor at a slow rate (about 0.2mL/min) until a stable baseline was achieved. The magnet was then placed under the sensor. The magnet was placed such that it generated a 20 field of force sufficient to capture magnetic beads on the surface of the sensor.

25 The processed sample was then circulated over the sensor. The bead solution was circulated for approximately 2 minutes at medium to fast flow rates (approximately 0.38mL/min). Due to the magnet, a high concentration of 30 beads was captured over the sensor surface.

The substrate (2mM PAPG, 100µl) was then circulated over the sensor at a slow flow rate (approximately 0.2mL/min). The flow was then stopped while substrate solution was over the sensor and the signal was measured with no flow for the desired period of time. The signal was

measured for about 90 to about 100 seconds for 60 seconds of useable data.

The beads were cleared from the sensor by removing the magnet from the proximity of the sensor and circulating 5 fresh ESB at a high flow rate (approximately 0.43mL/min) over the sensor. The addition of bubbles to the ESB flow was found to assist the clearance of the beads. The washing step generally took between about 45 and 60 seconds. Once the beads were washed out, fresh ESB was recycled over the 10 sensor until the baseline equilibrated. This step generally took about 30 seconds. The sensor was then ready for a new sample.

After coating the magnetic beads with p24, the beads were incubated for one minute with the serum to be tested 15 (primary incubation), washed, incubated for one minute with goat anti human beta-galactosidase IgG (secondary incubation) and washed. The procedure described above was then used to concentrate the beads over the sensor and the substrate was added.

20 Figure 2 is a graphical representation of the measured change in voltage over time. The first peak, starting at about t 11900 and ending at about t 12200 corresponds to the measurement of anti p24 in the low titer serum. The second peak, starting at about t 12500 and ending at about t 12800 25 corresponds to the measurement of anti p24 in the medium titer serum. The third peak, starting at about t 13200 and ending at about t 13500 corresponds to the measurement of anti p24 in the high titer serum.

The average slope was calculated from the data graph 30 (nAmp = y-axis; time (seconds) = x-axis) for data points acquired from the 20th second through the 100th second of measurement. Data was acquired at the rate of 2

observations per second and recorded as spreadsheet entries by the acquisition program (Origin Software). For the low titer serum the average slope was estimated to be 0.061, the average slope for the medium titer serum was estimated to be 5 0.112, and the average slope for the high titer serum was estimated to be 0.344. These values can be compared to the optical measurements obtained using a commercially available kit. The optical measurement provided values of 0.147, 0.291 and 0.495 for the low, medium and high titer serums 10 respectively. Advantageously, a tight correlation therefore was observed between the results obtained using the present invention and those obtained using commercially available systems. The results obtained using the present invention, however, required only a fraction of the time required for 15 the commercially available system and method.

Example 2

An experiment was conducted to find out the sensitivity of the systems and methods of the present invention under the conditions described below. Serial dilutions of human 20 serum having concentrations equivalent to 0, 15, 50, 100, 200, 400 and 800 mIU/ml anti-HBsAg were prepared. Magnetic beads (DYNABEADS M450) which had previously been coated with HBsAg were washed and resuspended in MBE.

For the primary incubation, the serum samples were 25 diluted 1:1 with MBE, and 25 μ l of each diluted sample was dispensed in a microtiter plate well. 5 \times 10⁶ coated beads in 25 μ l MBE were then added to each well. The samples were incubated for 2 minutes with gentle rocking at room temperature. The samples were then washed twice with MBE.

30 For the secondary incubation, 50 μ l of a 1:1000 dilution of goat anti human beta galactosidase conjugate in MBE was added to each well. The samples were incubated for 2

minutes with gentle rocking at room temperature. The samples were then washed twice with MBE, twice with PBST, once with ESB, and then resuspended in 250 μ l. The samples were then individually loaded onto the chip. PAPG 2mM in 5 ESB was then added to the system and the voltage in the sensor recorded.

Figure 3 is a graph plotting the slope of the kinetic measurement (nA/s) against the original concentration (mIU/ml) of HBsAg in the sample. The results indicate a 10 correlation having an R^2 equal to 0.8626. Qualitative results are obtainable for concentrations at least as low as 15 mIU/ml, with semi-quantitative results obtainable from 50 mIU/ml or greater under these conditions. As shown in Example 4 *infra*, more sensitive results may be obtained by 15 slightly varying the conditions.

Example 3

In this set of experiments, hepatitis B surface antigen (HBsAg) levels in human serum were measured. The measurement of dilutions corresponding to 0, 15, 50, 100, 20 200, 400 and 800mIU/ml were obtained using side by side matched conditions for all reagents. A direct comparison was made between the sensitivity of the methods and devices in accordance with the present invention and the devices and methods disclosed in WO 99/07879, which are at least as 25 sensitive and reliable as the colorimetric assay commercially available, to obtain a direct comparison between the system and method with and without the novel aspects of the present invention.

Preliminarily, DYNABEADS(M450) (4×10^8) were coated with 30 200 μ g of HBsAg (100 μ g of ad subtype obtained from human plasma and 100 μ g of ayw recombinant HBsAg) in a 850 μ l reaction volume following the same protocol used for p24 in

Example 1. In parallel, a substantially identical surface area of a microtiter plate was also similarly coated.

Sets of microbeads and microtiter plate wells were then incubated (primary incubation) with the different dilutions 5 of HBsAg serum samples for two minutes at room temperature. After two minutes the samples were removed and the different sets of microbeads and the wells of the microtiter plates were washed. The microbeads and microtiter plate wells were then subjected to a two minutes secondary incubation with 10 goat anti-human β -galactosidase. The conjugate was then removed and excess conjugate was washed off.

The microbead samples corresponding to the different dilutions of the sample were then individually measured by capturing the microbeads over the sensor, adding the 15 substrate solution and measuring the change in voltage over a period of 60 seconds. Figure 4 is graphic representation of the results obtained.

Similarly, the matched pairs measured in the microtiter plates were assessed by stopping the reaction after two 20 minutes, and measuring in a traditional manner the electrochemical generated. Figure 5 is a graphic representation of the results obtained.

As can be seen by comparing figure 4 to figure 5, the method and device of the present invention provides under 25 these conditions a linear dose response that can qualitatively detect as low as 200mIU/ml anti-HBsAg at 2 standard deviations uncertainty, with a linear dose response up to 800 mIU/ml. In contrast, the results obtained using the traditional method showed no statistically significant 30 difference between the samples, i.e., the traditional method under these conditions does not demonstrate a measurable dose dependent increase in electrochemical, and in fact the

traditional method under these conditions cannot qualitatively detect the analyte at a concentration below 800 mIU/ml.

The sensitivity and reliability of the method and device, in particular as demonstrated by the results obtained using the short primary and secondary incubations at room temperature was much greater than expected. These properties of the methods and devices of the present invention are valuable because they permit faster, cheaper, less cumbersome analysis of a sample.

Example 4

Having determined the unexpected and valuable properties of the system and method of the present invention, experiments were performed in order to optimize the procedure. In this experiment, the effect of the concentration of magnetic beads per sample to be analyzed was evaluated.

The experiment was generally set up as in Example 2. Serum samples having three concentrations (100 mIU/ml, 500 mIU/ml, 2000 mIU/ml) of HBsAg were tested using either 800,000 beads per sample, as in Example 2, or 5,000,000 beads per serum sample. The data was derived as in Example 2, and is shown graphically in Figure 6.

The data from the experiment indicates that the sensitivity of the device and method can be further greatly enhanced by increasing the number of magnetic beads per volume of sample. This presents a further advantage over the traditional method since it permits an increase in the surface area over which reactions can take place. As may be seen from Figure 6, a concentration of 100 mIU/ml can easily be detected using the larger amount of beads. Lower concentrations were not tested, but the linearity of the

response indicates that concentrations as low as 15 mIU/ml should be easily obtained with the increased number of beads.

As shown in Fig. 7, another embodiment of the invention comprises forming linearly disposed discrete solution compartments within a conduit. Each solution compartment may be defined by interposing a separator, such as a gas bubble, within a carrier fluid at predetermined points. In this manner, the carrier fluid may be divided up into solution compartments, each of which is formed or sandwiched between two opposing gas bubbles within the conduit. The conduit, such as an inert tube, may be parallel to a ground surface, vertical to a ground surface, or even at an angle thereto. Preferably, the conduit is vertically positioned relative to a ground surface.

Each solution compartment may contain a different composition of materials, such as a sample or a conjugate, to respectively define a sample solution compartment or a conjugate solution compartment. At least one of the solutions compartments contains an attractable bead coated with a recognition molecule to define a coated bead solution compartment.

In operation, each of the solution compartments are transported over time, from left to right as seen in Fig. 7, within the conduit via a peristaltic pump or the like. An attraction device, such as a magnet, electromagnet, or the like, is disposed about the conduit. The attraction device, when actuated, is capable of attracting one or more of the attractable beads for processing/testing. The attraction device preferably contains a sensor, or IDA chip as described in detail *supra*. The sensor is capable of measuring the manipulated and processed beads after they

have been transported through the conduit and/or subjected to the "conveyor belt" of discrete solution compartments.

Advantageously, as each of the solution compartments are transported within the conduit, due to the placement of 5 the attraction device, the attraction device is capable of selectively retaining at least some of the attractable beads within the conduit. In this manner, the attracted attractable beads are effectively separated from the carrier fluid. As the carrier fluid continues to flow through the 10 conduit, the next linearly disposed solution compartment can manipulate the temporarily restrained beads. For example, if the next linear solution compartment comprises a wash solution, the temporarily restrained beads will be washed. Similarly, if the solution compartment preceding the wash 15 solution compartment contains a substrate/carrier fluid, the bathed and temporarily restrained beads can be subjected to the substrate/carrier fluid within the conduit.

As is apparent to one of ordinary skill in the art, such a conduit arrangement allows for the implementation of 20 separate processing steps in an endless sequence that can be manipulated depending on the assay. The preferred linear order of the solution compartments, as illustrated in Fig. 7 from left to right, is as follows: a substrate/carrier fluid compartment, a conjugate/carrier fluid compartment, a 25 sample/carrier fluid compartment, and a bead compartment. Most preferably, a wash solution compartment separates each of the four identified material-containing solution compartments. Multiple attraction devices are also preferably used to facilitate improved processing 30 techniques.

In sum, the preferred operational steps of this embodiment of the invention, as illustrated in Fig. 7, are

as follows: (1) transporting a coated bead solution compartment to a first separation station having an actuatable attraction device; (2) actuating the attraction device to attract some of the coated beads in the coated bead solution compartment such that some of the coated beads are temporarily restrained within the first separation station and separated from the carrier fluid; (3) flowing a wash solution compartment into the first separation station; (4) flowing a sample/carrier fluid solution compartment over the attracted beads; (5) actuating the attraction device to release the temporarily restrained beads into the sample solution compartment; (6) flowing the sample/bead mixture from the first separation station preferably to a second separation station having an actuatable attraction device; (7) actuating the second attraction device to attract some of the coated beads such that some of the coated beads are temporarily restrained within the second separation station; (8) flowing a wash solution compartment into the second separation station; (9) flowing a conjugate/carrier fluid solution compartment over the attracted beads; (10) actuating the second attraction device to release the temporarily restrained beads into the conjugate/carrier fluid solution compartment; (11) flowing the conjugate/bead mixture from the second separation station to a third separation station that preferably has a third actuatable attraction device having a sensor; (12) actuating the third actuatable attraction device to attract some of the beads, or more specifically, some of the bead/antigen/antibody/enzyme complex, to the vicinity of the sensor; (13) flowing a wash solution compartment into the third separation station; (14) flowing a substrate/carrier fluid solution compartment over the attracted beads, which

in the presence of the enzyme is cleaved into a reporter molecule capable of exhibiting redox recycling, and (15) measuring the presence or amount of electrochemical with the sensor, wherein the sensor produces redox recycling of the 5 electrochemical.

Thus, devices and methods for detecting and quantitating analytes in a sample are disclosed. While embodiments and applications of this invention have been shown and described, it will be apparent to those skilled in 10 the art that many modifications are possible without departing from the inventive concepts herein. The invention, therefore is not to be restricted except in the spirit of the appended claims.

We claim:

1. An electrochemical reporter device comprising:

- (a) a chamber for receiving an analytical reaction including magnetic beads;
- 5 (b) a sensor within the chamber, the sensor for detecting electrochemical reporter molecules within the chamber and the sensor having a configuration such that reporter molecules capable of exhibiting redox recycling will undergo redox recycling if within the chamber; and
- 10 (c) an actuatable magnetic field generating device selectively positioned such that magnetic beads present within the chamber will be attracted to the surface of the chamber wherein the sensor is located.

2. The electrochemical reporter device of claim 1, the sensor being a microelectronic interdigitated array of electrodes with a distance between the electrodes of about

20 100 to about 800 nanometers.

3. The electrochemical reporter device of claim 2, the sensor being a microelectronic interdigitated array of electrodes having a distance between the electrodes of about

25 300 nanometers.

4. An electrochemical reporter system comprising:

- (a) a magnetic bead;
- (b) a first recognition molecule capable of
- 30 specifically binding an analyte in a structure restricted manner, the recognition molecule being linked to the magnetic bead;

- (c) an enzyme;
- (d) a coupling element, for coupling with specificity the enzyme to the recognition molecule/analyte complex or the analyte;
- 5 (e) a substrate which in the presence of the enzyme is cleavable into a reporter molecule capable of exhibiting redox recycling;
- (f) a sensor for detecting the electrochemical reporter molecule, said sensor having a configuration such that the reporter molecule will exhibit redox recycling; and
- 10 (g) a magnetic field generating device positionable such that the magnetic beads may be attracted to the vicinity of the sensor.

15

5. The electrochemical reporter system of claim 4, the sensor being a microelectronic interdigitated array of electrodes with a distance between the electrodes of between about 100 to about 800 nanometers.

20

6. The electrochemical reporter system of claim 5, the distance between the electrodes being about 300 nanometers.

25

7. The electrochemical reporter system of claim 4, the enzyme being capable of effecting the cleavage of a covalent bond of the substrate.

30

8. The electrochemical reporter system of claim 7, the enzyme being selected from the group consisting of α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -mannosidase, β -mannosidase, acid phosphatase, alkaline phosphatase and phosphodiesterase II.

9. The electrochemical reporter system of claim 4, the substrate being selected from the group consisting of p-aminophenyl- β -D-galactopyranoside, p-aminophenyl- α -D-galactopyranoside, p-aminophenyl- α -D-glucopyranoside, p-aminophenyl- β -D-glucopyranoside, p-aminophenyl- α -D-mannopyranoside, p-aminophenyl- β -D-mannopyranoside, p-aminophenylphosphate, and p-aminophenylphosphorylcholine.

10 10. The electrochemical reporter system of claim 4, the first recognition molecule being selected from the group consisting of a protein, a polypeptide, a nucleic acid, a nucleic acid analog, a hapten, immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, cell 15 adhesion molecules, receptors, non-biologic binding molecules and a hormone.

11. The electrochemical reporter system of claim 4, the coupling element comprising a second recognition molecule 20 coupled to an enzyme, the second recognition molecule being selected from the group consisting of a protein, a polypeptide, a nucleic acid, a nucleic acid analog, a hapten, immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, cell adhesion 25 molecules, receptors, non-biologic binding molecules and a hormone.

12. The electrochemical reporter system of claim 4, the substrate being cleaved into at least one component 30 comprising para-aminophenol.

13. The electrochemical reporter system of claim 4, the sensor being a microelectronic interdigitated array of electrodes having width between about 100 and about 800 nanometers and spaced between about 100 and about 800
5 nanometers from each other.

14. An assay for detecting or quantitating a specific analyte in a sample comprising the steps of:

- a) a primary incubation, wherein magnetic beads coated with a
10 first recognition molecule that specifically binds an analyte are incubated with a sample;
- b; a secondary incubation, wherein the magnetic beads are incubated with a conjugate comprising an enzyme and a second recognition molecule that specifically binds the
15 analyte, or the analyte/recognition molecule complex;
- c) capturing the magnetic beads with a magnet over a sensor capable of producing redox recycling of an electrochemical capable of undergoing redox recycling;
- d) adding a substrate, said substrate in the presence of the
20 enzyme being cleaved into an electrochemical capable of undergoing redox recycling; and
- e) detecting the presence or measuring the amount of electrochemical present in the solution with said sensor.

25 15. The assay of claim 14, the primary incubation lasting less than 10 minutes.

16. The assay of claim 14, the secondary incubation lasting less than 10 minutes.

30 17. The electrochemical reporter system of claim 14, the first recognition molecule being selected from the group

consisting of a protein, a polypeptide, a nucleic acid, a nucleic acid analog, a hapten, immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, cell adhesion molecules, receptors, non-biologic binding 5 molecules and a hormone.

18. The electrochemical reporter system of claim 14, the second recognition molecule being selected from the group consisting of a protein, a polypeptide, a nucleic acid, a nucleic acid analog, a hapten, immunoglobulin, fragments of immunoglobulin, non-immunoglobulin binding proteins, cell adhesion molecules, receptors, non-biologic binding 10 molecules and a hormone.

15 19. The electrochemical reporter system of claim 14, the enzyme being capable of effecting the cleavage of a covalent bond of the substrate.

20. The electrochemical reporter system of claim 19, the enzyme being selected from the group consisting of α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -mannosidase, β -mannosidase, acid phosphatase, alkaline phosphatase and phosphodiesterase II.

25 21. The electrochemical reporter system of claim 14, the substrate being selected from the group consisting of p-aminophenyl- β -D-galactopyranoside, p-aminophenyl- α -D-galactopyranoside, p-aminophenyl- α -D-glucopyranoside, p-aminophenyl- β -D-glucopyranoside, p-aminophenyl- α -D-30 mannoside, p-aminophenyl- β -D-mannopyranoside, p-aminophenylphosphate, and p-aminophenylphosphorylcholine.

22. The electrochemical reporter system of claim 14 wherein the substrate is cleaved into at least one component comprising para-aminophenol.

5 23. The electrochemical reporter system of claim 14 wherein the sensor is a microelectronic interdigitated array of electrodes having width between about 100 and about 800 nanometers and spaced between about 100 and about 800 nanometers from each other.

10

24. An electrochemical immunoassay for detecting an analyte in a sample comprising the steps of:

15 (a) having linked to a magnetic bead an antigen with an antibody specific for an analyte bound to the antigen, the antibody being coupled to an enzyme or having a coupling element for being specifically coupled to an enzyme;

20 (b) contacting the magnetic bead/antigen/antibody/enzyme complex with a sample to be analyzed;

(c) collecting the magnetic bead/antigen/antibody/enzyme complex;

25 (d) attracting the magnetic bead/antigen/antibody/enzyme complex to the vicinity of a sensor;

(e) adding a substrate to the collected magnetic bead/antigen/antibody/enzyme complex, the substrate in the presence of the enzyme being cleavable into a reporter molecule capable of exhibiting redox recycling; and

30 (f) measuring the presence or amount of reporter molecule with the sensor, the sensor being an

interdigitated array of electrodes capable of producing redox recycling of the reporter molecule.

5 25. An electrochemical assay for detecting a specific analyte in a sample comprising the steps of:

(a) having a recognition molecule linked to a magnetic bead, said recognition molecule capable of specifically binding the analyte in a structure

10 restricted manner;

(b) contacting the magnetic bead with a sample to be analyzed;

(c) coupling with specificity an enzyme to the recognition molecule or the analyte;

15 (d) attracting the magnetic bead/recognition molecule/analyte/enzyme conjugate complex to the vicinity of a sensor with a device capable of generating a magnetic field;

(e) adding a substrate, which in the presence of the enzyme is cleaved into a reporter molecule capable of exhibiting redox recycling; and

20 (f) measuring the presence or amount of electrochemical with the sensor, wherein the sensor is an interdigitated array of electrodes capable of producing redox recycling of the electrochemical.

25

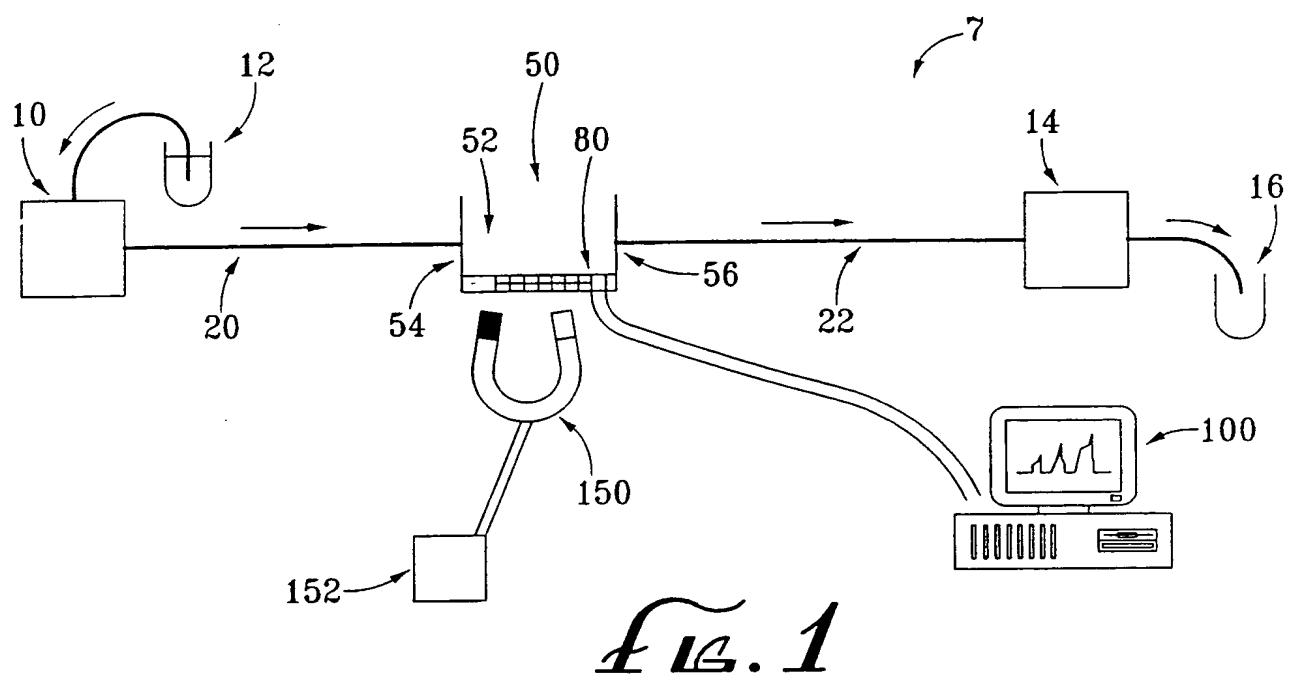
26. A kit for detecting or measuring an analyte in a sample the kit comprising

30 i) magnetic beads pre-coated with a first recognition molecule specific for the analyte;

ii) a second recognition molecule, specific for the analyte or the first recognition molecule/analyte complex, the second recognition molecule being conjugated to an enzyme;
iii) a substrate which in the presence of the enzyme
5 generates an electrochemical capable of redox recycling.

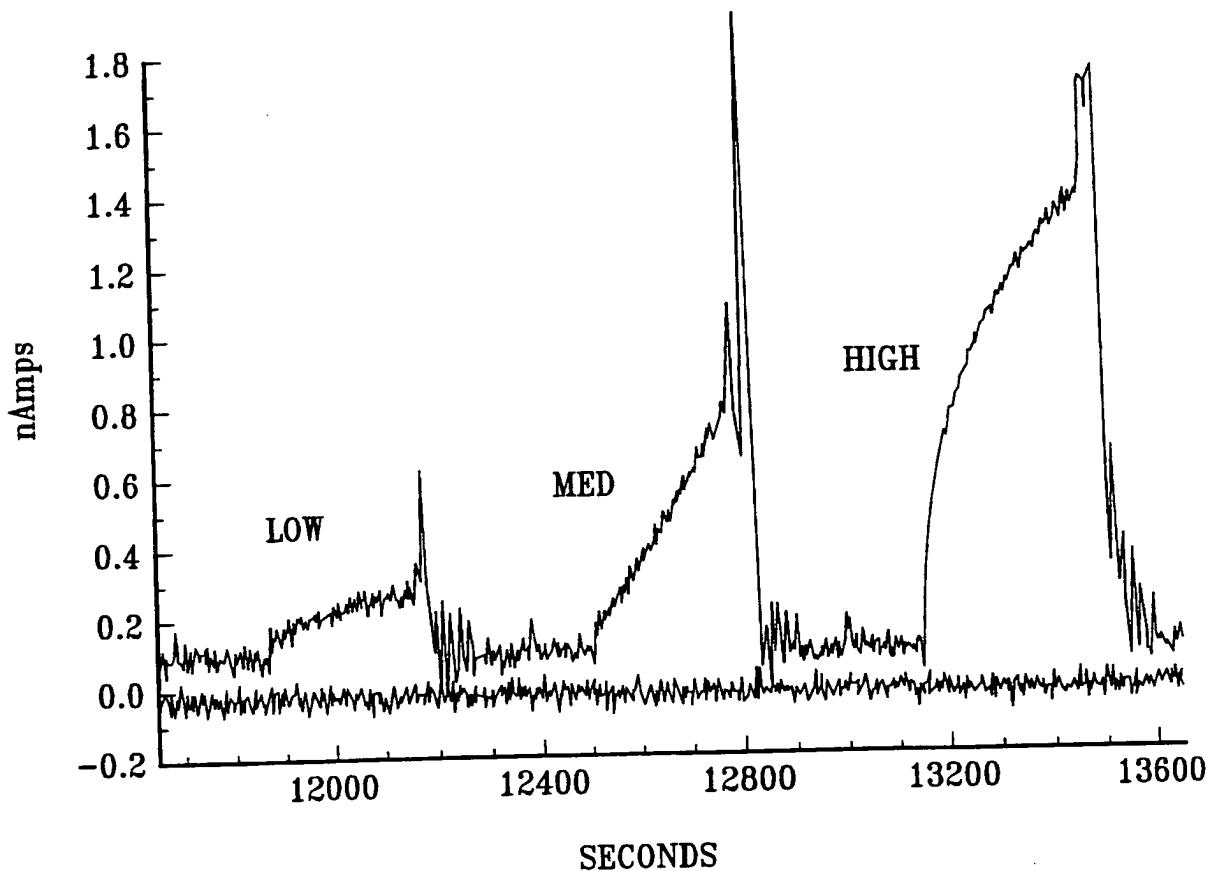
27. The kit of claim 26 further comprising
iv) a single use electrochemical sensor module.

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P24 COATED MAGNETIC BEADS

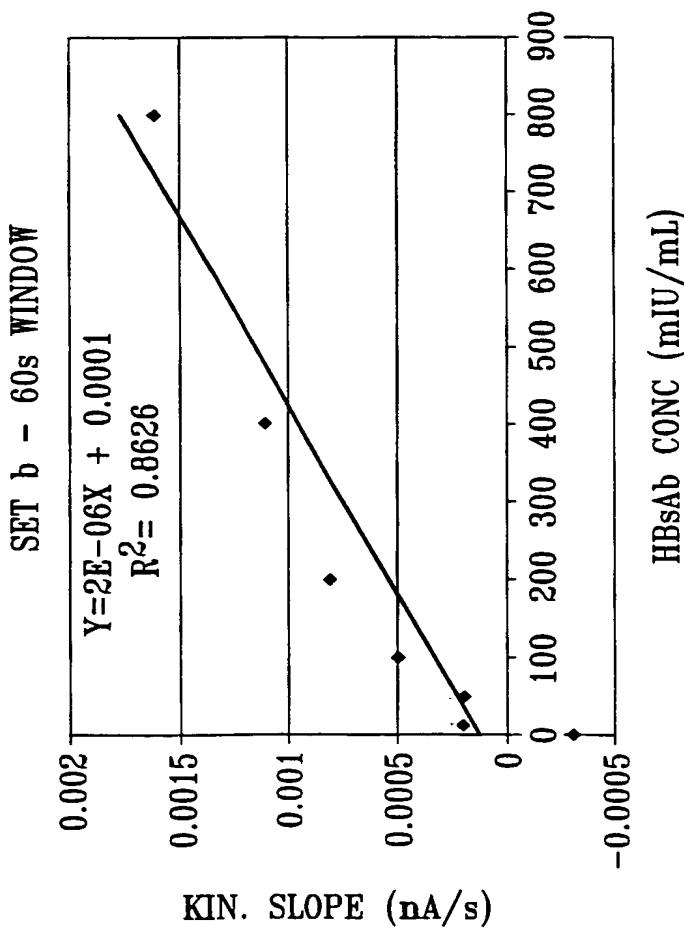
*FIG. 2*

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	+20 TO +80 SEC WINDOW	+20 S THRU END OF CURVE	SLOPE (nA/SEC)
SAMPLE (mIU/mL)			(nA/SEC)
SET B	0	-0.0003	nA
	15	0.0002	0.0001
	50	0.0002	0.0002
	100	0.0005	0.0002
	200	0.0008	0.0004
	400	0.0011	0.0007
	800	0.0016	0.0012

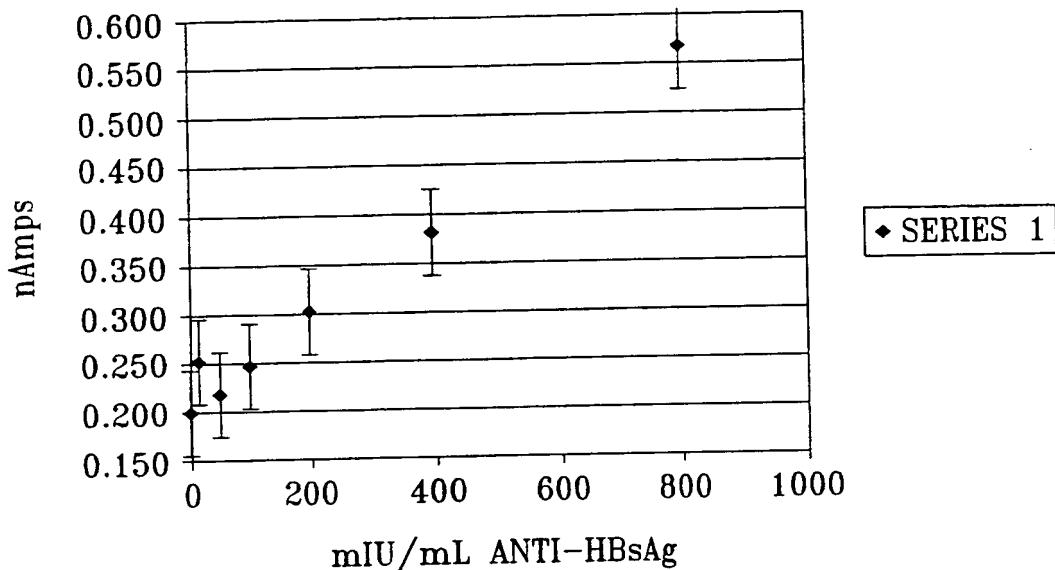
SENSITIVITY - TO 15 mIU/mL
 FOR QUALITATIVE RESULT.
 - TO 50 mIU/mL FOR
 SEMI-QUANTITATIVE RESULT.

Fig. 3



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FIG. 4



STD/ERROR BARS @ 0.015

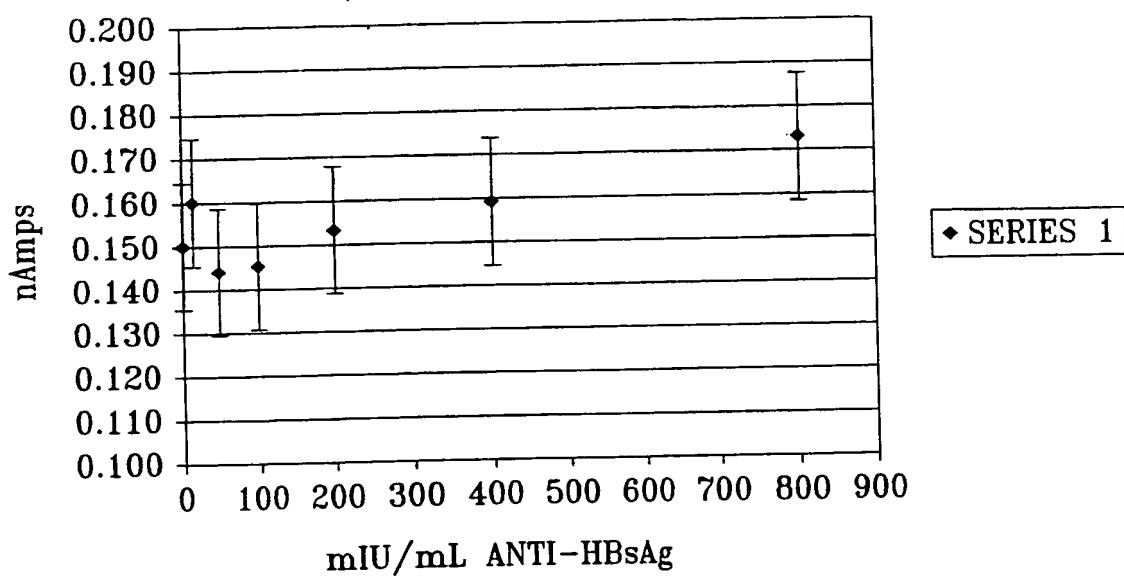


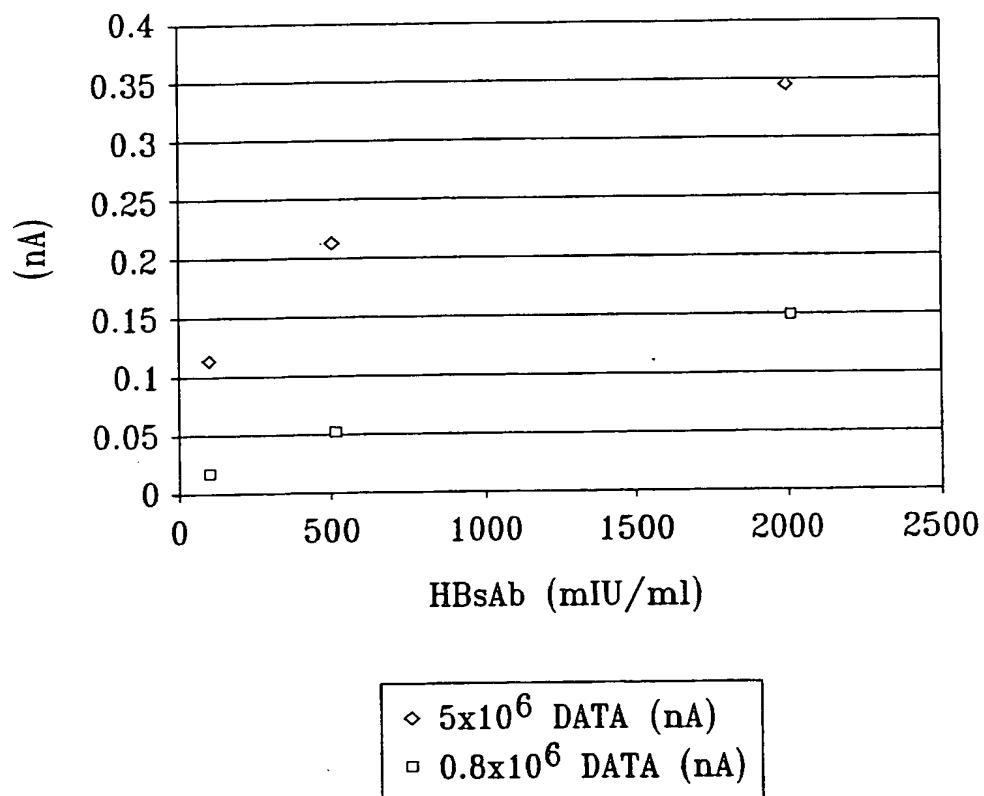
FIG. 5

SUBSTITUTE SHEET (RULE 26)

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HBsAb (mIU/ml)	5×10^6 DATA (nA)	0.8×10^6 DATA (nA)
----------------	---------------------------	-----------------------------

2000	0.3439	0.1472
500	0.2158	0.052
100	0.118	0.0177



◆ 5×10^6 DATA (nA)
□ 0.8×10^6 DATA (nA)

Fig. 6

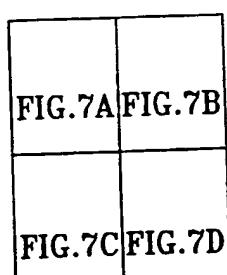
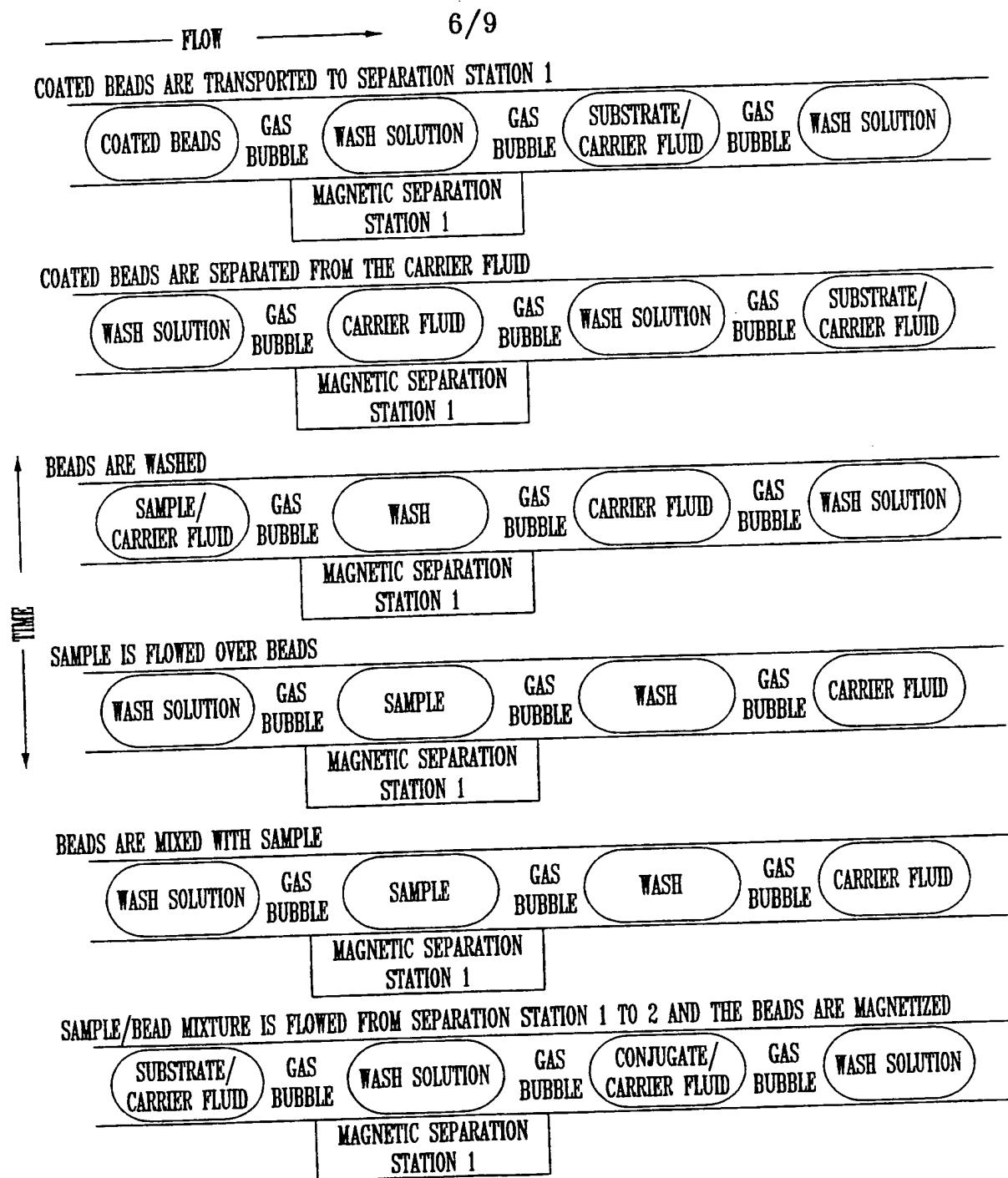


FIG. 7A

FIG. 7

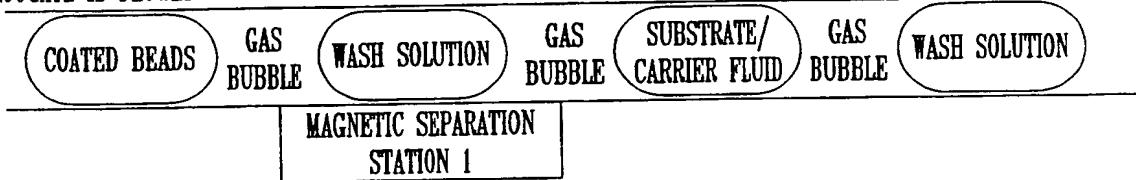
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GAS BUBBLE	CONJUGATE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	SAMPLE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION
MAGNETIC SEPARATION STATION 2						IDA CHIP	MAGNETIC SEPARATION STATION 3
GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	CONJUGATE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	SAMPLE/ CARRIER FLUID
MAGNETIC SEPARATION STATION 2						IDA CHIP	MAGNETIC SEPARATION STATION 3
GAS BUBBLE	SUBSTRATE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	CONJUGATE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION
MAGNETIC SEPARATION STATION 2						IDA CHIP	MAGNETIC SEPARATION STATION 3
GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	SUBSTRATE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	CONJUGATE/ CARRIER FLUID
MAGNETIC SEPARATION STATION 2						IDA CHIP	MAGNETIC SEPARATION STATION 3
GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	SUBSTRATE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	CONJUGATE/ CARRIER FLUID
MAGNETIC SEPARATION STATION 2						IDA CHIP	MAGNETIC SEPARATION STATION 3
GAS BUBBLE	SAMPLE	GAS BUBBLE	WASH	GAS BUBBLE	CARRIER FLUID	GAS BUBBLE	WASH SOLUTION
MAGNETIC SEPARATION STATION 2						IDA CHIP	MAGNETIC SEPARATION STATION 3

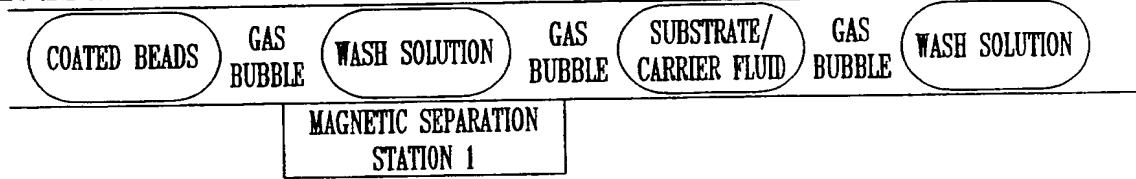
FIC. 7B

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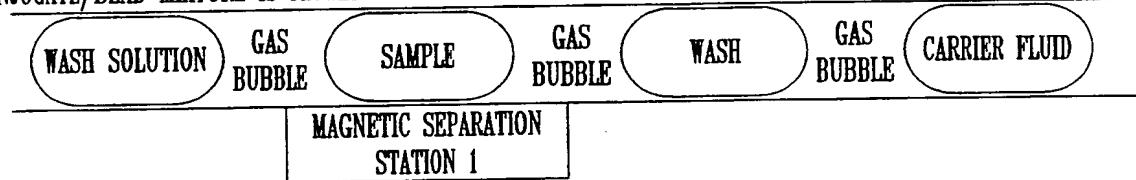
CONJUGATE IS FLOWED OVER BEADS



BEADS ARE MIXED WITH CONJUGATE



CONJUGATE/BEAD MIXTURE IS FLOWED FROM STATION 2 TO 3 OVER IDA CHIP AND THE BEADS ARE MAGNETIZED



CONJUGATE/BEAD MIXTURE IS MAGNETIZED TO IDA CHIP

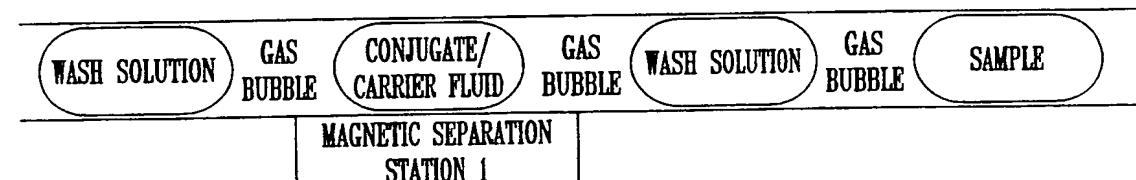
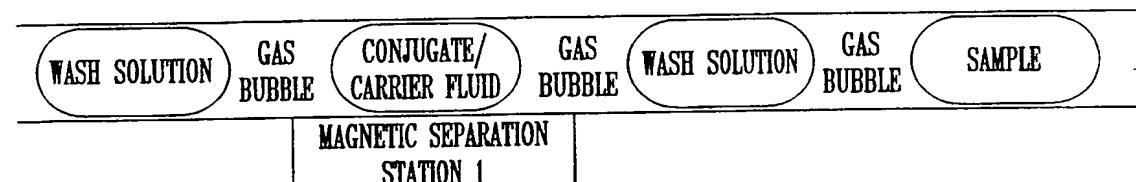
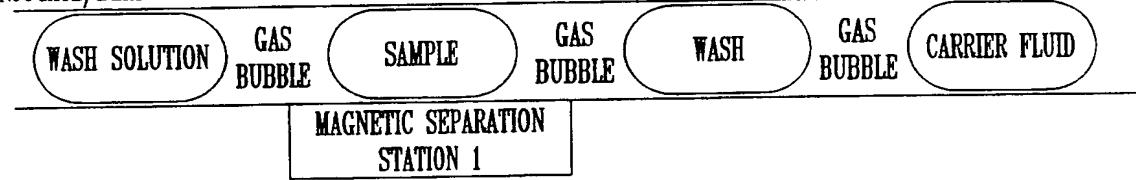


FIG. 7C

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GAS BUBBLE	CONJUGATE	GAS BUBBLE	WASH	GAS BUBBLE	CARRIER FLUID	GAS BUBBLE	WASH
MAGNETIC SEPARATION STATION 2						IDA CHIP	
						MAGNETIC SEPARATION STATION 3	
GAS BUBBLE	CONJUGATE	GAS BUBBLE	WASH	GAS BUBBLE	CARRIER FLUID	GAS BUBBLE	WASH
MAGNETIC SEPARATION STATION 2						IDA CHIP	
						MAGNETIC SEPARATION STATION 3	
GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	SUBSTRATE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	CONJUGATE
MAGNETIC SEPARATION STATION 2						IDA CHIP	
						MAGNETIC SEPARATION STATION 3	
GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	SUBSTRATE/ CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	CONJUGATE
MAGNETIC SEPARATION STATION 2						IDA CHIP	
						MAGNETIC SEPARATION STATION 3	
GAS BUBBLE	WASH	GAS BUBBLE	CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	SUBSTRATE
MAGNETIC SEPARATION STATION 2						IDA CHIP	
						MAGNETIC SEPARATION STATION 3	
GAS BUBBLE	WASH	GAS BUBBLE	CARRIER FLUID	GAS BUBBLE	WASH SOLUTION	GAS BUBBLE	SUBSTRATE
MAGNETIC SEPARATION STATION 2						IDA CHIP	
						MAGNETIC SEPARATION STATION 3	

15.7D

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/03485

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N27/327 G01N33/543 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, COMPENDEX, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GEHRING A G ET AL: "Enzyme-linked immunomagnetic electrochemical detection of <i>Salmonella typhimurium</i> " JOURNAL OF IMMUNOLOGICAL METHODS, NL, ELSEVIER SCIENCE PUBLISHERS B.V., AMSTERDAM, vol. 195, no. 1, 9 September 1996 (1996-09-09), pages 15-25, XP004021249 ISSN: 0022-1759 page 16, column 1, paragraph 1 -page 16, column 2, paragraph 2 figure 2 ---	1, 4, 7-12, 14-22, 26, 27
Y	page 16, column 1, paragraph 1 -page 16, column 2, paragraph 2 figure 2 ---	24, 25 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

Date of mailing of the international search report

5 July 2000

13/07/2000

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PCT/US 00/03485

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	O NIWA ET AL: "Small-volume voltammetric detection of 4-aminophenol with interdigitated array electrodes and its application to electrochemical enzyme immunoassay" ANALYTICAL CHEMISTRY, US, AMERICAN CHEMICAL SOCIETY, COLUMBUS, vol. 65, no. 11, 1 January 1993 (1993-01-01), pages 1559-1563, XP002082750 ISSN: 0003-2700 abstract page 1560, column 1, paragraph 2 ---	24, 25
X	WEETALL H H ET AL: "A SIMPLE INEXPENSIVE DISPOSABLE ELECTROCHEMICAL SENSOR FOR CLINICAL AND IMMUNO-ASSAY" BIOSENSORS, vol. 3, no. 1, 1987, pages 57-64, XP000922945 ISSN: 0265-928X page 61, paragraph 2 -page 62, paragraph 1 figure 1 ---	1, 4, 7, 8, 10, 11, 14-20, 24-27
X	SANTANDREU M ET AL: "Development of electrochemical immunosensing systems with renewable surfaces" BIOSENSORS & BIOELECTRONICS, 1 JAN. 1998, ELSEVIER, UK, vol. 13, no. 1, pages 7-17, XP000922856 ISSN: 0956-5663 page 11 -page 12 figure 4 ---	1, 4, 7, 10, 11, 14-19, 26, 27
X	EP 0 859 229 A (GIST BROCADES BV) 19 August 1998 (1998-08-19) page 4, line 18 - line 28 page 4, line 49 - line 57 examples 4, 5 ---	1, 4, 7, 8, 10, 11, 14-20, 26, 27
X	WO 86 05815 A (GENETICS INT INC) 9 October 1986 (1986-10-09) claim 21 -----	1, 4, 10, 11, 14-18, 26, 27

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/03485

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0859229	A 19-08-1998	AU	5301598 A	13-08-1998
WO 8605815	A 09-10-1986	AU EP	5667186 A 0216844 A	23-10-1986 08-04-1987



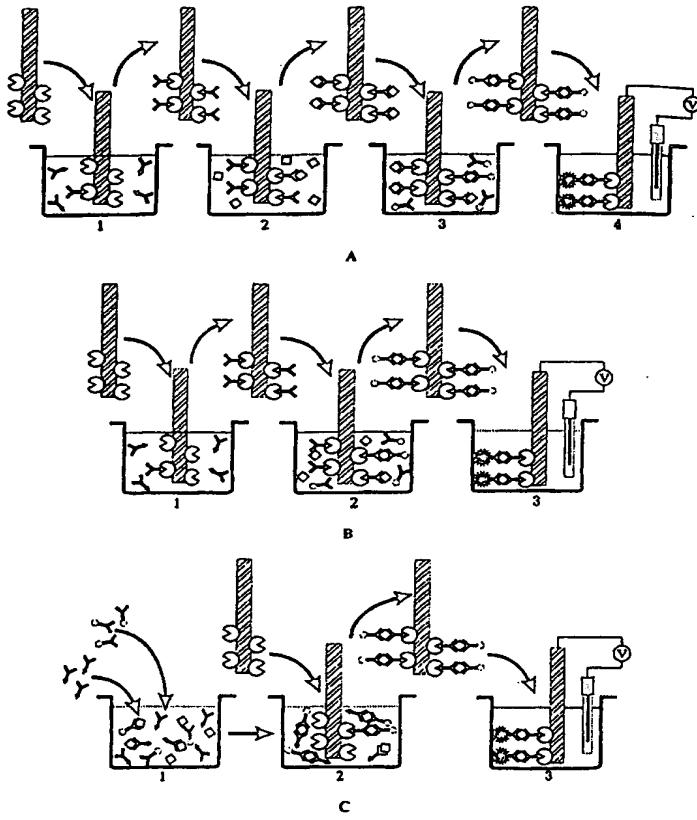
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : G01N 33/543, 27/327, C12Q 1/28, 1/58, 1/68		A1	(11) International Publication Number: WO 00/11473 (43) International Publication Date: 2 March 2000 (02.03.00)
(21) International Application Number: PCT/GB99/02785			(74) Agent: BOULT WADE TENNANT; 27 Furnival Street, London EC4A 1PQ (GB).
(22) International Filing Date: 24 August 1999 (24.08.99)			
(30) Priority Data: 98116346 24 August 1998 (24.08.98)	RU		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(71) Applicant (for all designated States except US): SENSOR-TECH LIMITED [-/-]; Don Road, P.O. Box 301, St. Helier, Jersey JE4 8UG (GB).			
(71) Applicant (for LR only): BALDOCK, Sharon, Claire [GB/GB]; 27 Furnival Street, London EC4A 1PQ (GB).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): FARMAKOVSKI, Dmitri Alexandrovich [RU/RU]; Building 48, Flat 46, Kastanaevskaya Street, Moscow, 1121108 (RU). MILANOVSKI, Yevgeni Yurevich [RU/RU]; Building 48, Flat 46, Kastanaevskaya Street, Moscow, 1121108 (RU). CHERKASOV, Vladimir Rurikovich [RU/RU]; Building 48, Flat 46, Kastanaevskaya Street, Moscow, 1121108 (RU). BIRYUKOV, Yuri Sergeyevich [RU/RU]; Building 48, Flat 46, Kastanaevskaya Street, Moscow, 1121108 (RU). LEONARDOVA, Olga [RU/CA]; 1830-11 Avenue S.W. #401, Calgary, Alberta T3C 0N6 (CA).			

(54) Title: METHOD OF ELECTROCHEMICAL ANALYSIS OF AN ANALYTE

(57) Abstract

A sensing electrode for use in methods of electrochemical analysis comprising an electrically conducting electrode coated with an electroconductive polymer membrane having immobilised therein or adsorbed thereto adaptor molecules avidin, streptavidin, anti-fitc antibodies through which the sensing electrode can be made specific for an analyte under test by the binding of receptors specific for the analyte.



6A3614P-6t.-a9te

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METHOD OF ELECTROCHEMICAL ANALYSIS
OF AN ANALYTE

The invention relates to methods of
5 electrochemical detection of analytes and to sensing
electrodes for use in methods of electrochemical
detection.

Electrochemical analysis of analytes, such as
various antigens, antibodies, DNA molecules etc, in
10 biological fluids using biosensors is one of the most
promising and attractive methods of instrument
analysis. The sustained interest and large number of
publications in this field are explained by a number
of basic advantages of the method, namely high
15 sensitivity, simplicity and the use of relatively
simple and inexpensive equipment.

It is known in the art to construct biosensor
devices based on the use of electroconductive polymer
films, such as polypyrrole or polythiophene, which
20 transduce a chemical signal associated with the
presence of an analyte into a measurable electrical
signal (see [1] and [2]).

EP-A-0 193 154 describes an electrode for use in
electrochemical detection, the electrode being coated
25 with a polypyrrole or polythiophene film.

Bioreceptors complementary to the analyte to be tested
are adsorbed onto the surface of the electroconductive
polymer film after polymerisation. WO 89/11649
describes an alternative process for the production of
30 polymeric electrodes for use in electrochemical
assays. In this process bioreceptor molecules having
the desired binding specificity are incorporated into
a film of electroconductive polymer during
polymerisation. Using the processes described in EP-
35 A-0 193 154 and WO 89/11649 for each given assay it is

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necessary to synthesise a different sensing electrode having immobilized bioreceptors capable of specifically binding to the analyte for which one wants to test.

5 The applicants' published application PCT/GB98/00548 describes a potentiometric method of electrochemical analysis using an electrochemical sensing electrode comprising a metallic potentiometric electrode again coated with a layer of
10 electroconductive polymer containing immobilised bioreceptor molecules which bind specifically to the analyte under test. The presence of analyte is indicated by a change in surface charge of the sensing electrode upon binding of analyte to the immobilised
15 bioreceptors. The analyte detection procedure is carried out by first assembling an electrochemical cell comprising the sensing electrode and a reference electrode connected together by means of a measuring device immersed in a working buffer solution of fixed pH. A base value of potential difference between the sensing electrode and the reference electrode is recorded, the sensing and reference electrodes are then brought into contact with a solution of higher ionic strength suspected of containing the analyte but
20 with pH the same as the working buffer and potential difference is again recorded. The sensing and reference electrodes are finally transferred to clean working buffer and potential is again recorded. The change in potential difference between the sensing
25 electrode and the reference electrode resulting from a change in ionic strength of the buffer at constant pH in the presence of analyte is proportional to the concentration of analyte.

30 As shown in references [13, 14, 15, 16] and in [3], the response (amount and rate of potential

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change) of the sensing electrode with polymer film containing bioreceptors to a step-change in ionic strength of the ambient solution (so-called "ion-step" procedure) is to a large extent determined by the 5 charge on the polymer film. Apart from the material from which it is made, the polymer film charge is determined by the charge of the receptor molecules bound in it. If the receptor charge changes as a result of an affinity reaction with a specific 10 analyte, the response of the sensing electrode will also change as a result of the ion-step procedure carried out after contact between the sensing electrode and the test fluid. It should be noted that, because of the amphoteric nature of the majority 15 of analytes, the receptor charge depends on the pH of the solution and it is therefore very important to maintain a constant pH of the solution during the ion-step procedure.

Thus, in the previously described methods, based 20 on measurement of the change in response of the sensing electrode to the ion-step procedure carried out before and after contact between the sensing electrode and test fluid, it is possible to make a determination as to the presence in the test fluid of 25 analyte specific to the receptors bound on the sensing electrode. In the ideal case, the variation in the charge of the receptors in the membrane and, hence, the change in the sensing electrode response is directly proportional to the concentration in the test 30 fluid of analyte specific to the receptors bound on the sensing electrode. However, in real conditions, the charge of the same analyte can vary considerably, which produces inconsistent quantitative results. Moreover, the affinity reactions are not always 35 accompanied by a change in receptor charge. This

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normally occurs when testing small or non-charged antigens [14].

In summary, the shortcomings of the prior art methods of electrochemical detection based on the use of electroconductive polymer electrodes include complexity and limited amenability to industrialisation of the sensing electrode manufacturing process, inconsistency of the characteristics of the sensing electrodes obtained, limited ability to store the sensing electrodes without loss of performance. In addition, the previously described protocols for electrochemical detection, particularly the method described in PCT/GB98/00548, are of limited use for the detection of small and non charged molecules or molecules whose isoelectric point is close to the isoelectric point of the receptors immobilised on the surface of the sensing electrode.

The present invention provides a method of electrochemical analysis of an analyte in a sample which is to a large extent free of the shortcomings inherent in the methods described above in that it widens the scope of application by virtue of the ability to analyse small and non-charged molecules, provides strictly quantitative results, makes the electrode manufacturing process more amenable to industrial production methods, increases the productivity of the analysis, improves reproducibility and therefore enhances reliability of the results obtained.

Thus, in a first aspect the invention provides a sensing electrode for use in methods of electrochemical detection of an analyte, the sensing electrode comprising an electrically conductive electrode coated with an electroconductive polymer

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with adaptor molecules selected from the group consisting of avidin, streptavidin, anti-FITC antibodies and a molecule capable of binding to at least one class of receptor molecules immobilised therein or adsorbed thereto.

One of the principal problems inherent in electrochemical analysis methods using sensing electrodes is the problem of retention over time of the native properties of the receptors fixed on the sensing electrodes. Relative progress has been achieved in this field only for a limited number of enzyme sensing electrodes [7]. For the majority of electrochemical sensing electrodes using refined receptors known in the literature [8, 9, 10], their useful storage life is simply not stated. The retention of the native properties of immobilised receptors is particularly critical where antibodies are used as the receptors, which is attributable to their inherent high degree of conformational variability.

In contrast, it is known that antibodies and other biomolecules retain their useful properties over very long periods of time when stored in the form of concentrated solutions; therefore the problem of prolonged storage of the sensing electrodes without loss of working characteristics may be overcome by rapid immobilisation of receptors before use or even during the electrochemical detection procedure.

This problem is solved in the declared invention by use of so-called adaptor molecules which are immobilized in or adsorbed to the electroconductive polymer. The purpose of the adaptor molecules is to link receptor molecules specific to the analyte under test to the surface of the sensing electrode. As will be discussed below, with the use

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of adaptor molecules it is possible to temporally separate steps in the production of the sensing electrodes. Thus, it is feasible to manufacture the electrodes with immobilised/adsorbed adaptor molecules, store them for an extended period of time and then fix the specific receptors onto the electrode either before or during the electrochemical analysis. With the selection of appropriate adaptor molecules it is also possible to manufacture 'universal' sensing electrodes containing adaptor molecules capable of binding to a whole range of different receptor molecules. Specificity for the analyte under test is conferred on the 'universal' sensing electrode simply by binding to the adaptor molecules receptors of the appropriate specificity. It is therefore no longer necessary to incorporate receptors of the desired specificity during the electrodeposition process.

The proteins avidin and streptavidin are preferred for use as adaptor molecules. Avidin, a protein obtained from raw eggs, consists of four identical peptide sub-units, each of which has one site capable of bonding with a molecule of the co-factor biotin. Biotin (vitamin H) is an enzyme co-factor present in very minute amounts in every living cell and is found mainly bound to proteins or polypeptides. The ability of biotin molecules to enter into a binding reaction with molecules of avidin or streptavidin (a form of avidin isolated from certain bacterial cultures, for example *Streptomyces aviation*) and to form virtually non-dissociating "biotin-avidin" complexes during this reaction (dissociation constant ~ 10^{-15} Mol/l) is well known [11, 12].

Investigations carried out by the authors of the declared invention have shown that avidin and

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streptavidin immobilised in an electroconductive polymer film, retain their native properties for an extended period of time (at least one year and possibly longer) and can be used throughout this period to link with biotin conjugated receptors. Techniques which allow the conjugation of biotin to a wide range of different molecules are well known in the art. Thus sensing electrodes with immobilised avidin or streptavidin can easily made specific for a given analyte merely by binding of the appropriate biotinylated receptors.

Although avidin and streptavidin are the preferred adaptor molecules it is within the scope of the invention to use alternative adaptor molecules, in particular molecules capable of specifically binding to at least one class of receptor molecules. Included within this group of alternative adaptor molecules are protein A, protein G and lectins. These molecules all share the ability to bind to at least one class of receptor molecules, by which is meant that they are able to specifically bind to a common binding site motif which is present in each member of a group of receptor molecules, the dissociation constant for the binding interaction being less than 10^{-8} Mol/l. By way of example, protein A (a 42kD polypeptide isolated from *Staphylococcus aureus* or obtained by recombinant DNA technology) binds to immunoglobulins, particularly IgG, from a wide range of mammalian species at the Fc region; and protein G (IgG Fc receptor Type III, see Bjorck, L. and Kronvall, G., J. Immunol., 133, 969 (1984)) also binds to the Fc region of IgG molecules from a wide range of mammalian species. Lectins are proteins which bind to sugar moieties which may be present on glycoproteins or carbohydrates. Each type of lectin has specificity for a given sugar moiety and

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thus will be able to bind a range of glycoproteins or complex carbohydrates carrying the correct sugar moiety.

5 In a still further embodiment anti-FITC antibodies can be used as the adaptor molecules. In this embodiment, the specificity of the sensing electrode for analyte can be conferred by binding to the anti-FITC antibodies FITC labelled receptors of the appropriate specificity.

10 The use of adaptor molecules in/on the electroconductive polymer film also considerably improves the reliability of the results obtained during electrochemical analysis by reducing non-specific interactions of the components of the test 15 solution during contact with the sensing electrode, which is linked to the blocking of the free surface of the electroconductive polymer by adaptor molecules. The use of adaptor molecules also increases the technical efficiency of the sensing electrode 20 manufacturing process, for example by eliminating the need for an additional surface blocking procedure.

25 The potentiometric sensing electrodes of the invention are inexpensive to manufacture and so for convenience can be produced in a disposable format, intended to be used for a single electrochemical detection experiment or a series of detection experiments and then thrown away. The invention further provides an electrode assembly including both a sensing electrode and a reference electrode required 30 for electrochemical detection. As will be discussed below, suitable reference electrodes include silver/silver chloride and calomel electrodes. Conveniently, the electrode assembly could be provided as a disposable unit comprising a housing or holder 35 manufactured from an inexpensive material equipped

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with electrical contacts for connection of the sensing electrode and reference electrode.

The sensing electrodes of the invention can be used in a wide range of electrochemical analysis procedures, including (but not limited to) double antibody sandwich assays for antigens, double antigen sandwich assays for antibodies, competitive assays for antigens, competitive assays for antibodies, serological assays for the determination of human antibodies (e.g. Rubella IgG antibodies using labelled antihuman antibodies) and IgM assays (e.g. IgM-Rubella antibodies).

In a second aspect the invention provides a method of producing a sensing electrode for use in methods of electrochemical detection of an analyte, the sensing electrode comprising an electrically conductive electrode coated with an electroconductive polymer with adaptor molecules selected from the group consisting of avidin, streptavidin and a molecule capable of binding to at least one class of receptor molecules immobilized therein, the method comprising the steps of:

(a) preparing an electrochemical polymerisation solution comprising monomeric units of the electroconductive polymer and adaptor molecules,
(b) immersing the electrode to be coated in the electrochemical polymerisation solution, and
(c) applying a cyclic electric potential between the electrode and the electrochemical polymerisation solution to coat the electrode by electrochemical synthesis of the polymer from the solution, said cyclic electric potential being applied for at least one full cycle.

The invention further provides a method of producing a sensing electrode for use in methods of

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electrochemical detection of an analyte in a sample,
the electrode comprising an electrically conductive
electrode coated with an electroconductive polymer
with adaptor molecules selected from the group
5 consisting of avidin, streptavidin and a molecule
capable of binding to at least one class of receptor
molecules adsorbed thereto, the method comprising
steps of:

10 (a) preparing an electrochemical polymerisation
solution comprising monomeric units of the
electroconductive polymer,

(b) immersing the electrode to be coated in the
electrochemical polymerisation solution,

15 (c) applying a cyclic electric potential
between the electrode and the electrochemical
polymerisation solution to coat the electrode by
electrochemical synthesis of the polymer from the
solution, said cyclic electric potential being
applied for at least one full cycle; and

20 (d) contacting the coated electrode with a
solution comprising adaptor molecules such that the
adaptor molecules are adsorbed onto the
electroconductive polymer coating of the electrode.

According to the methods of the invention a film
25 of electroconductive polymer is deposited onto the
surface of an electrically conductive electrode by
electrochemical synthesis from a monomer solution.
The electrically conductive electrode is preferably a
standard potentiometric electrode possessing metallic
30 or quasi-metallic conductivity which is stable in
aqueous media. As will be illustrated in the
examples included herein, electrodeposition of the
electroconductive polymer film is carried out using a
solution containing monomers, a polar solvent and a
35 background electrolyte. Pyrrole, thiophene, furan or

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aniline are the preferred monomers. Deionised water is preferably used as the polar solvent.

As is well known to persons skilled in the art, electroconductive polymers are often doped at the 5 electrochemical synthesis stage in order to modify the structure and/or conduction properties of the polymer. A typical dopant anion is sulphate (SO_4^{2-}) which is incorporated during the polymerisation process, neutralising the positive charge on the 10 polymer backbone. Sulphate is not readily released by ion exchange and thus helps to maintain the structure of the polymer. In the present invention it is preferred to use dopant anions having maximum 15 capability for ion exchange with the solution surrounding the polymer in order to increase the sensitivity of the electrodes. This is accomplished by using a salt whose anions have a large ionic radius as the background electrolyte when preparing the electrochemical polymerisation solution.

20 Suitable salts whose anions have large ionic radius include sodium dodecyl sulphate and dextran sulphate. The concentration of these salts in the electrochemical polymerisation solution is varied according to the type of test within the range 0.005 25 - 0.05 M.

As reported in a number of papers [4, 5], the ease with which ion exchange takes place and the rapidity with which ion equilibrium is attained for 30 electroconductive polymers immersed in a solution are essentially dependent on the size of the anti-ion introduced at the electrodeposition stage: the larger the ionic radius of the anti-ion, the more readily ion-exchange reactions take place and the more rapidly a state of equilibrium is reached. This is 35 directly linked to the value and rate of change of

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the potential of the "metal electrode -
electroconductive polymer" system in response to
variation in the ion composition of the solution [6].

5 The electroconductive polymer membrane performs
a dual function, serving both to bind the receptor to
the surface of the sensing electrode, and to render
the sensing electrode sensitive to variations in the
composition of the buffer solution. In particular,
changes in the composition of the buffer solution
10 which affect the redox composition of the
electroconductive polymer result in a corresponding
change in the steady state potential of the sensing
electrode.

15 Adaptor molecules may either be immobilized in
the electroconductive polymer film at the
electrochemical synthesis stage by adding adaptor
molecules to the electrochemical polymerisation
solution or may be adsorbed onto the surface of the
electroconductive polymer film after electrochemical
20 polymerisation. In the former case, a solution of
adaptor molecules may be added to the
electrodeposition solution immediately before the
deposition process. The deposition process works
optimally if the storage time of the finished
25 solution does not exceed 30 minutes. Depending on
the particular type of test, the concentration of
adaptor molecules in the solution may be varied in
the range 5.00 - 100.00 μ g/ml. Procedures for
electrodeposition of the electroconductive polymer
30 from the solution containing adaptor molecules are
described in the examples included herein. On
completion of electrodeposition process, the sensing
electrode obtained may be rinsed successively with
deionised water and 0.01 M phosphate-saline buffer
35 solution and, depending on the type of test, may then

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be placed in a special storage buffer solution containing microbial growth inhibitors or bactericidal agents (e.g gentamicin), or dried in dust-free air at room temperature.

5 Where the adaptor molecules are to be adsorbed after completion of the electrodeposition process the following protocol may be used (although it is hereby stated that the invention is in no way limited to the use of this particular method), the sensing electrode
10 is first rinsed with deionised water and placed in freshly prepared 0.02M carbonate buffer solution, where it is held for 15-60 minutes. The sensing electrode is then placed in contact with freshly-prepared 0.02M carbonate buffer solution containing
15 adaptor molecules at a concentration of 1.00 - 50.00 µg/ml, by immersing the sensing electrode in a vessel filled with solution, or by placing a drop of the solution onto the surface of the sensing electrode. The sensing electrode is incubated with the solution
20 of adaptor molecules, typically for 1-24 hours at +4°C. After incubation, the sensing electrode is rinsed with deionised water and placed for 1-4 hours in a 0.1M phosphate-saline buffer solution. Depending on the type of test, the sensing electrode may then
25 be placed either in a special storage buffer solution containing microbial growth inhibitors or bactericidal agents, or dried in dust-free air at room temperature.

When the adaptor molecules are avidin or streptavidin, the above-described methods of the invention for producing a sensing electrode may optionally comprise a further step of contacting the coated electrode with a solution comprising specific receptors conjugated with biotin such that said
35 biotinylated receptors bind to molecules of avidin or

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streptavidin immobilised in or adsorbed to the electroconductive polymer coating of the electrode via a biotin/avidin or biotin/streptavidin binding interaction.

5 Research carried out both by the authors of the declared invention, and by others [12], has shown that the biotinylation of receptors under optimal conditions does not alter their properties (affinity, storage qualities, etc.) compared with their non-biotinylated equivalents.

10 Conjugation of biotin with the corresponding receptors, a process known to those skilled in the art as biotinylation, can be carried out using one of the known procedures, for example as described in 15 [12]. In addition, a number of ready-made preparations of biotinylated antibodies of different specificity are commercially available, e.g. Anti-Human IgG or Anti-Human IgA goat biotin-labelled antibodies made by Calbiochem-Novabiochem, USA.

20 One of the significant advantages of using biotinylated receptors is the ability to vary the specificity of the sensing electrode, by producing a reaction between the avidin or streptavidin bound on the sensing electrode and the corresponding biotinylated receptors. As discussed previously, the 25 sensing electrode with bound avidin/streptavidin is in effect a 'universal sensing electrode' and specificity to the desired molecules under test is conferred by the binding of the appropriate biotinylated receptors. To make the sensing 30 electrode with bound avidin or streptavidin specific to the analyte under test, a reaction is carried out between the avidin or streptavidin bound on the sensing electrode with biotinylated receptors, for 35 which purpose the sensing electrode is brought into

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contact with a solution of the latter at room temperature, either by immersing the sensing electrode in a vessel filled with solution, or by placing drop of solution on the sensing electrode surface (concentration of biotinylated receptors in the solution is generally 0.1 - 100 μ g/ml; contact time 3-15 minutes).

The receptor molecules can be any molecule capable of specifically binding to another molecule (an analyte). Suitable types of receptors include monoclonal and polyclonal antibodies, chimaeric antibodies, fragments of antibodies which retain the ability to recognise antigen (e.g. Fab and Fab2 fragments), recombinant proteins and fragments thereof, synthetic peptides, antigens, single-strand DNA, RNA or PNA molecules, hormones, hormone receptors, enzymes, chemical compounds etc.

As discussed above, the electrochemical detection methods known in the art using potentiometric sensing electrodes are of limited use in the detection of small and non-charged antigens. In order to overcome this problem, and to obtain strictly quantitative results, use may be made of secondary receptors or competing molecules conjugated with a charge label.

Accordingly, in a further aspect the invention provides a method of electrochemical detection of an analyte in a sample, which method comprises the steps of:

- 30 (a) providing a sensing electrode having an electroconductive polymer coating, the coating having immobilised therein or adsorbed thereto receptors which specifically bind to the desired analyte to be detected in the sample;
- 35 (b) treating the sensing electrode by immersion

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in a test solution comprising the sample so that said desired analyte binds to said immobilised or adsorbed receptors;

5 (c) contacting the sensing electrode with a solution comprising secondary receptors capable of binding to said analyte at a site spatially distinct from the site of binding to the immobilised or adsorbed receptors, said secondary receptors being conjugated with a charge label;

10 (d) monitoring the electric potential difference between the treated sensing electrode and a reference electrode when both are immersed in an electrolyte; and

15 (e) monitoring the electric potential difference between the sensing electrode and a reference electrode following a change in the ionic strength of the electrolyte at constant pH.

The affinity reaction steps of the above-described method are equivalent to a standard sandwich assay well known to those skilled in the art. The sandwich format of analysis is particularly useful for the detection of polyvalent antigens in which case the receptors and labelled secondary receptors used in the test are antibodies which bind to different, spatially distinct epitopes on the antigen. The sandwich format can also be used where the antigen carries two or more identical epitopes which are spatially separated. In this latter case, the receptors and labelled secondary receptors used in the test may be antibodies of identical specificity.

30 It is also within the scope of the invention to perform the electrochemical analysis in a competitive assay format. Therefore, the invention also provides a method of electrochemical detection of an analyte

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in a sample comprising the steps of:

(a) providing a sensing electrode having an electroconductive polymer coating, the coating having immobilised therein or adsorbed thereto receptors which are capable of binding to the desired analyte to be detected in the sample;

(b) treating the sensing electrode by immersion in a test solution comprising the sample so that said desired analyte binds to said immobilised or adsorbed receptors;

(c) contacting the sensing electrode with a solution comprising competing molecules capable of binding to said immobilised or adsorbed receptors, said competing molecules being conjugated with a charge label;

(d) monitoring the electric potential difference between the treated sensing electrode and a reference electrode when immersed in an electrolyte; and

(e) monitoring the electric potential difference between the sensing electrode and a reference electrode following a change in the ionic strength of the electrolyte at constant pH.

In this competitive electrochemical assay the competing molecules could be labelled analyte or labelled structural analogs of the analyte which are capable of binding to the same analyte binding site on the immobilized/adsorbed receptors (see Fig 5B and Fig 7B). The use of labelled analyte as the competing molecule is particularly preferred for the detection of small analyte molecules (e.g. digoxin, as described in the examples included herein). Alternatively, the competing molecule may bind to a different site on the immobilized/adsorbed receptor.

For example, if the immobilized receptor is an

antibody, the competing molecule could be an anti-immunoglobulin antibody (preferably Fab-specific) or even an anti-idiotype antibody of the appropriate specificity (see Fig 5A and Fig 7A).

5 As would be readily understood by persons skilled in the art, with reference to Figures 5A, 5B, 7A and 7B of the present application, the competitive detection methods are usually dependent on their being an excess of receptor sites on the surface of
10 the sensing electrode. Those receptors which do not bind analyte will be available for binding to the competing molecule. Assuming that the total number of receptor sites remains constant, the amount of bound competing molecule will be inversely
15 proportional to the amount of analyte present.

In order to transduce the chemical signal associated with the concentration of the analyte into a measurable electrical signal the charge label which is conjugated to the secondary receptors or competing
20 molecules can be any charge label having the following properties:

(i) carries a net charge (positive or negative) at the pH of the electrolyte of part (d); and
25 (ii) the magnitude of this charge changes in response to a change in the ionic strength of the electrolyte at constant pH.

30 Preferably the charge label is highly charged, i.e. has a net charge at the pH of the electrolyte of part (d) of greater than one electrostatic unit (e). Suitable charge labels include gold, ferrocene and latex microspheres. The magnitude of the charge on the charge label affects the redox composition of the electroconductive polymer coating on the sensing electrode such that the step change in ionic strength
35 between steps (d) and (e) results in a detectable

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change in potential difference between the sensing electrode and the reference electrode. The charge label is only brought into close proximity of the electroconductive polymer on formation of receptor/analyte/secondary receptor complexes (sandwich assay) or receptor/competing molecule complexes (competitive assay). The use of a charge label thus makes it possible to obtain correct qualitative results and considerably extends the spectrum of testable analytes by virtue of the ability to test small and non-charged analytes.

Latex microspheres are the preferred type of charge label conjugated with the secondary receptors or competing molecules. Conjugation with latex microspheres may be carried out using one of the known techniques, for example as described in [17] or [18], or using special commercially available kits for the conjugation of antibodies with latex microspheres, for example "Carbodiimide Kit for Carboxylated Beads" made by Polysciences Inc., USA, following the protocol supplied by the manufacturer. Certain ready-made latex conjugates are commercially available from specialist manufacturers, e.g. Polysciences Inc., USA.

As an alternative to the use of a charge label, it is also possible to perform electrochemical detection procedures equivalent to those described above using secondary receptors or competing molecules conjugated with an enzyme labels in order to transduce the chemical signal associated with the concentration of the analyte into a measurable electrical signal.

Accordingly, the invention further provides a method of electrochemical detection of an analyte in a sample, which method comprises the steps of:

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(a) providing a sensing electrode having an electroconductive polymer coating, the coating having immobilized therein or adsorbed thereto receptors which are capable of binding to the desired analyte to be detected in the sample;

(b) treating the sensing electrode by immersion in a test solution comprising the sample so that the said analyte binds to said immobilized or adsorbed receptors;

(c) contacting the sensing electrode with a solution comprising secondary receptors capable of binding to said analyte at a site spatially distinct from the site of binding to immobilized or adsorbed receptors, said secondary receptors being conjugated with an enzyme;

(d) monitoring the electric potential difference between the treated sensing electrode and a reference electrode when both are immersed in an electrolyte; and

(e) monitoring the electric potential difference between the sensing electrode and a reference electrode following exposure to an electrolyte comprising the substrate for said enzyme.

Also within the scope of the invention is the corresponding competitive method of electrochemical detection comprising the steps of:

(a) providing a sensing electrode having an electroconductive polymer coating, the coating having immobilized therein or adsorbed thereto receptors which are capable of binding to the desired analyte to be detected in the sample;

(b) treating the sensing electrode by immersion in a test solution comprising the sample so that the said desired analyte binds to said immobilized or adsorbed receptors;

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(c) contacting the sensing electrode with a solution comprising competing molecules capable of binding to said immobilized or adsorbed receptors, said competing molecules being conjugated with an enzyme;

5 d) monitoring the electric potential difference between the treated sensing electrode and a reference electrode when both are immersed in an electrolyte; and

10 e) monitoring the electric potential difference between the sensing electrode and a reference electrode following exposure to an electrolyte comprising the substrate for said enzyme.

In one embodiment of the above-described methods the enzyme conjugated to the secondary receptors or competing molecules is capable of converting a substrate which directly affects the redox composition of the electroconductive polymer coating of the sensing electrode into a product which has no detectable effect on the redox composition of the electroconductive polymer. An example of such an enzyme is horseradish peroxidase.

In an alternative embodiment, the enzyme conjugated to the secondary receptors or competing molecules is capable of converting a substrate which has no detectable effect on the redox composition of the electrochemical polymer coating of the sensing electrode to a product capable of directly or indirectly affecting the redox composition of the electroconductive polymer. One way in which the product of the enzymic reaction may indirectly affect the redox composition of the electroconductive polymer is by causing a change in the pH of the electrolyte (for this embodiment the pH of the electrolyte is not buffered). An example of an

enzyme which generates such a product is urease.

In a still further embodiment, the enzyme conjugated to the secondary receptors or the competing molecules is capable of converting a 5 product which has no detectable effect on the redox composition of the electroconductive polymer coating of the sensing electrode into a product which is a substrate for a second enzyme, the action of the second enzyme generating a second product which 10 directly or indirectly affects the redox composition of the electroconductive polymer.

In all embodiments the conjugated enzyme is brought into close proximity of the electroconductive polymer by formation of receptor/analyte/secondary 15 receptor complexes (sandwich format) or by formation of receptor/competing molecule complexes (competitive format).

The conjugation of secondary receptors or competing molecules with enzyme labels may be 20 performed by any of the techniques known in the art (see, for example [19]). Use can also be made of widely available commercial preparations of conjugates of receptors of different specificity with different enzyme labels.

All of the above methods of electrochemical 25 detection can be performed using any type of receptor capable of specifically binding to another molecule (an analyte). Suitable types of receptors include monoclonal and polyclonal antibodies, chimaeric 30 antibodies, fragments of antibodies which retain the ability to recognise antigen (e.g. Fab and Fab2 fragments), recombinant proteins and fragments thereof, synthetic peptides, antigens, single-strand DNA, RNA or PNA molecules, hormones, hormone 35 receptors, enzymes, chemical compounds etc.

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Regardless of whether the secondary receptors or competing molecules are conjugated with a charge or enzyme label, the maximum degree of specificity and sensitivity for all of the detection methods of the invention is achieved by performing the affinity reactions (i.e. steps (a) to (c)) in a 'sequential' format. This is particularly so when the analyte under test is a polyvalent antigen (i.e. the sandwich assay). In the sequential format the sensing electrode with bound receptors is first brought into contact with a test solution comprising the sample to be tested for the presence of the analyte. As used herein the term 'sample' includes within its scope any material which it is desired to test for the presence of analyte, including biological fluids such as whole blood, serum, plasma, urine, lymph, cerebrospinal fluid or semen, environmental fluids, materials used or produced in the food and drink industry or a dilution or extract of any of the above. The sample may also comprise a solution or extract of a solid material. The container used for the test solution may be the well of a microtiter plate, a micro-centrifuge tube or any other vessel of suitable size. The volume of test solution will generally be 5-200 μ l depending on the geometrical dimensions of the sensing electrode. The contact time between the sensing electrode and test solution is typically 3-30 minutes at 15-40°C with or without continuous mixing.

Following contact with the test solution the sensing electrode is transferred to a vessel containing solution of labelled secondary receptors. The vessel and volume of solution used are similar to those used for contact between the sensing electrode and test solution. The

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concentration of labelled secondary receptors or labelled competing molecules in the solution is typically 1-100 µg/ml depending on the required sensitivity of the test. Contact is made for 3-30 5 minutes at 15-40°C with or without continuous mixing.

As an alternative to the 'sequential' format it is possible to substantially reduce the total test time and simplify the test procedure by performing steps (b) and (c) simultaneously by contacting the 10 sensing electrode with test solution to which has been added the appropriate labelled secondary receptors or labelled competing molecules for a contact time of about 5-60 minutes. The 15 concentration of labelled secondary receptors or labelled competing molecules added to the test solution is typically 1-100 µg/ml depending on their type, specific features and required sensitivity of the test.

To eliminate possible non-specific interactions 20 between the components of biological fluids under test and the surface of the sensing electrode, and also non-specific adsorption of labelled secondary receptors or labelled competing molecules onto the surface of the sensing electrode, which will distort 25 the results obtained, various blocking agents may be added to the solution of labelled secondary receptors or labelled competing molecules. Suitable blocking agents include bovine serum albumin (0.5% - 5%), 30 human serum albumin (0.5 - 5wt.%), dilute normal human or animal serum (5-10vol.%), gelatin (10-50 vol.%), etc. In so doing, interaction of the labelled secondary receptors or labelled competing molecules with the sensing electrode is accompanied by simultaneous blocking of any free surface of the 35 sensing electrode.

In all of the detection methods of the invention use can be made of sensing electrodes containing immobilized/adsorbed adaptor molecules. In particular, the receptor molecules may be attached to the surface of the sensing electrode via biotin/avidin, biotin/streptavidin, protein A/antibody, protein G/antibody, FITC/anti-FITC or lectin/sugar binding interactions.

The use of 'universal' sensing electrodes containing adaptor molecules allows the detection methods to be performed in a 'one-pot' format. In this embodiment, the affinity reactions are performed in a homogeneous solution, providing maximum contact between the interacting molecules and ensuring maximum sensitivity and minimum duration of the test.

In this case, the solution of receptors and the solution of labelled secondary receptors or labelled competing molecules are added simultaneously or sequentially to the test solution comprising the sample suspected of containing the analyte in a single reaction vessel. The concentrations of receptors and labelled secondary receptors or labelled competing molecules in the test solution are typically 0.1 - 100 µg/ml and 1 - 100 µg/ml respectively. The test solution is then incubated at 15-40°C for 5-60 minutes with or without continuous mixing to allow the binding reactions to take place. The sensing electrode containing the appropriate adaptor molecules is then brought into contact with the test solution, either by immersion into the vessel containing the test solution, or by placing a drop of test solution on the surface of the sensing electrode. The contact time between the sensing electrode and test solution is typically 3-30 minutes at 15-40°C. Measurement of the amount of analyte

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bound on the sensing electrode is then performed using the "ion-step" procedure or by adding the appropriate enzyme substrate depending on whether the secondary receptors or competing molecules are 5 labelled with a charge or enzyme label.

Once all the affinity reaction steps are completed, an electrochemical measuring cell is assembled by bringing the sensing electrode and a reference electrode, connected by a measuring 10 instrument, into contact with an electrolyte solution (also referred to herein as a working solution) and the measuring device is used to record the sensing electrode potential relative to the reference electrode over a fixed time period. Commercially 15 available reference electrodes of suitable size, or electrodes purpose-designed for implementation of the declared invention, may be used as the reference. The measuring instrument is a standard potentiometric measuring instrument or potentiostat. PC-compatible 20 electronic measuring instruments purpose designed for implementation of the declared invention and controlled by custom software can also be used.

For convenience the sensing electrode and reference electrode can be linked to the measuring 25 instrument by means of a special holder equipped with electrical contacts for connection of the sensing electrode and reference electrode and connected to the measuring instrument by a cable or other means. A holder integral with the measuring instrument could 30 also be used, making it possible to miniaturise the measuring system in terms of its overall dimensions.

Aqueous buffer solutions are used as the working 35 solution : phosphate-saline, Tris-HCl, carbonate bicarbonate, acetate, borate, etc. The volume of working solution in the electrochemical cell is

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typically between 50 and 5000 μ l depending on the geometrical dimensions of the sensing electrode. The container for the buffer solution may be any suitably sized vessel in a material with minimal adsorption properties, e.g. the well of a standard microtiter plate. Another embodiment of the declared invention is a variant in which a low-volume (< 1cm³) flow-through cell is used in conjunction with an integral holder for the sensing electrode and reference electrode, through which buffer solution can be pumped by means of a peristaltic pump or other means.

The potential of the sensing electrode relative to the reference electrode potential is recorded for a fixed time period using a chart recorder connected to a potentiometric measuring device or potentiostat, or by means of a special program where PC-compatible electronic instrumentation is used. In the latter case, the program measures the sensing electrode potential relative to the reference electrode potential at pre-determined time intervals (typically every 3-5 seconds for a total of 10-100 seconds) and displays the results in the form of points on the coordinates "sensing electrode signal - time". Recording of sensing electrode potential relative to the reference electrode potential is carried out to determine the background potential value V_0 of the sensing electrode, and also to evaluate the background potential drift (γ) of the sensing electrode, which is calculated by linearisation of the curve "sensing electrode signal-time" obtained using the least squares method.

If the secondary receptors or competing molecules are conjugated with a charge label, the amount of analyte bound to the sensing electrode is

evaluated by changing the ionic strength of the electrolyte solution at constant pH, the so-called "ion-step" procedure.

In the ion-step procedure the ionic strength of the electrolyte solution may be modified (upwards or downwards) either by transferring the holder complete with sensing electrode and reference electrode from the initial working solution into a second working solution of the same composition but with a different 10 ionic strength, or by adding a buffer solution of different (higher or lower) ionic strength directly to the working solution in which the sensing electrode and reference electrode are immersed. If a flow through cell is used, the ionic strength of the 15 electrolyte solution can be modified by expelling the initial working solution from the cell using a buffer solution of different ionic strength.

Working solutions having different ionic strengths may be achieved by having different 20 concentrations of salts, e.g. KCl, Na₂SO₄, etc., the use of which is based on the fact that they dissociate completely when added to the solution and do not bias the pH of the solution. The concentration 25 of salts in the working solution ranges from 0.01 to 0.1 M.

If the secondary receptors or competing molecules are conjugated with an enzyme it is not necessary to perform the "ion-step" procedure. Instead, the composition of the working solution is modified by adding a suitable substrate for the 30 enzyme. To this end, either the holder complete with sensing electrode and reference electrode can be transferred from the vessel containing the initial working solution to a vessel 35 containing working solution plus substrate, or the

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substrate solution can be added directly to the original working solution in which the sensing electrode and reference electrode are immersed. If a flow-through cell is used, the composition of the 5 working solution can be modified by expelling the initial working solution from the cell using a working solution containing substrate.

Substrates which may be used include ABTS ({2,2'-Azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]}), TMB (3, 3, 5, 5'-Tetraethylbenzidine) , DAB (3, 3' Diaminobenzidine) (where the enzyme label is peroxidase), urea (where the enzyme label is urease), p-NPP (p-Nitrophenyl Phosphate), BCIP (5-bromo-4-chloro-3-indolylphosphate) (where the enzyme label is 15 alkaline phosphatase).

The variation in sensing electrode potential relative to the reference electrode potential in response to a step change in ionic strength of the working solution or addition of an enzyme substrate 20 is recorded for a fixed time period using a measuring instrument. Again, the recording is made either using a chart recorder connected to a potentiometric measuring device or potentiostat, or by means of a special program where PC-compatible electronic 25 instrumentation is used. In the latter case, the program measures the sensing electrode potential relative to the reference electrode potential at pre-determined time intervals (typically every 3-5 seconds) and displays the results in the form of 30 points on the coordinates "sensing electrode signal - time". Depending on the particular type of test, the time taken to record the variation in sensing electrode potential relative to reference electrode potential varies between 30 and 600 seconds.

35 In the case where the ionic strength of the

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buffer solution is changed, the curve obtained for the variation in sensing electrode potential relative to the reference electrode potential usually takes the form of a parabola, and represents the response of the sensing electrode to the change in ionic strength of the buffer solution, which is modulated by the total charge (isoelectric point) of the 5 electroconductive polymer film.

If the analysis is performed as a sandwich 10 assay, the variation in total charge of the polymer film is directly proportional to the quantity of the analyte under test. However, if the analysis is performed as a competitive assay, the variation in 15 total charge of the polymer film is generally inversely proportional to the quantity of analyte under test.

On completion of this stage in the procedure, 20 the final value V_2 of sensing electrode potential relative to reference electrode potential is determined. The following quantitative characteristics of the change in sensing electrode potential relative to the reference electrode potential can then be calculated:

25 1. the area (integral) described by the curve obtained for the change in sensing electrode potential relative to reference electrode potential, S_2 :

$$30 S_2 = \int_{T1}^{T2} f_2(t) dt$$

where: $T2-T1$ = total time period for recording of background potential drift or potential relative 35 to the reference electrode; f_2 - curve of

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"sensing electrode potential in millivolts
versus time"; t - current recording time;

and

5

2. difference in millivolts between the background
and final potential of the sensing electrode:

10

$$\delta = V_2 - V_1$$

15

Based on the quantitative characteristics of the
variation in sensing electrode potential in response
to a change in ionic strength or composition of the
working solution, a determination is made as to the
quantitative content of target analyte in the
test solution.

20

Using the values for γ , S_2 , and/or δ obtained as
described above it is possible to re-calculate the
values to allow for the zero line drift γ , yielding
the values S_2' and/or δ' , on the basis of which a
determination is made of the quantity of target
analyte in test solution. The corrected values S_2'
and δ' may be compared with a calibration curve of
"analytical result versus amount of target analyte".
As would be readily apparent to persons skilled in
the art, data for construction of a calibration curve
can be obtained in a manner similar to the procedure
described above using a range of test solutions
containing known amounts of the target analyte.

25

In a still further aspect the invention provides
a method of electrochemical detection of an analyte
in a sample, which method comprises the steps of:

(a) providing a sensing electrode comprising

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an electrically conductive electrode coated with a layer of electroconductive polymer with avidin or streptavidin immobilized therein or adsorbed thereto, said avidin or streptavidin molecules being attached to receptor molecules capable of binding the analyte to be detected attached via a biotin/avidin or biotin/streptavidin binding interaction;

5 (b) contacting the sensing electrode with a test solution comprising the sample so that said 10 desired analyte binds to said immobilized or adsorbed receptor molecules;

(c) monitoring the potential of the sensing electrode relative to a reference electrode when both are immersed in an electrolyte; and

15 (d) monitoring the potential difference of the sensing electrode relative to the reference electrode following a change in the ionic strength or composition of the electrolyte at constant pH.

This method of electrochemical detection is of use where the binding of the target analyte to the receptor is causes a change in charge on the surface of the sensing electrode which is sufficiently large to be measurable without the need for a separate charge or enzyme label. In particular, this method is useful in the electrochemical detection of nucleic acids. Hybridisation of target nucleic acids to nucleic acid probes (e.g. oligonucleotides) attached to the surface of the sensing electrode is accompanied by a change in charge sufficient large to be detectable by the "ion-step" procedure. There is thus no need to use secondary receptors or competing molecules conjugated with charge label. Materials suspected of containing specific nucleic acids (e.g. biological fluids) may commonly be subjected to an 30 amplification step (e.g. PCR) before being subjected 35

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to the detection procedure. It is therefore within the scope of the invention to perform the electrochemical detection of specific nucleic acids on samples which have been subjected to an 5 amplification procedure.

The present invention will be further understood with reference to the following non-limiting Examples together with the accompanying Figures in which the principal stages of its implementation are 10 depicted.

Fig. 1A: schematically illustrates a sensing electrode consisting of a potentiometric electrode (1) coated with a layer of electroconductive polymer 15 (2) with avidin or streptavidin molecules (3) immobilized in the polymer layer.

Fig. 1B: schematically illustrates a sensing electrode consisting of a potentiometric electrode 20 (1) coated with a layer of electroconductive polymer (2) with avidin or streptavidin molecules (3) adsorbed onto the layer of polymer.

Fig. 2A: illustrates the process of rendering the 25 sensing electrode specific to an analyte (in this case antigens, medium or high molecular weight substances) through a binding reaction between the avidin or streptavidin immobilized in the polymer layer and antibodies (4) conjugated with biotin (5).

Fig. 2B: illustrates the process of rendering the 30 sensing electrode specific to the analyte under test (in this case antibodies) through a binding reaction between avidin or streptavidin immobilized in the polymer layer and antigen (6) conjugated with biotin 35

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(5).

Fig. 2C: illustrates the process of rendering the sensing electrode specific to the analyte under test (in this case DNA molecules) through a binding reaction between avidin or streptavidin immobilized in the polymer layer and a DNA probe (7) conjugated with biotin (5).

Fig. 3A: shows the placement of the sensing electrode in contact with a test solution containing antigens (6) specific to biotinylated antibodies immobilized on the sensing electrode.

Fig. 3B: shows placement of the sensing electrode in contact with a test solution containing antibodies (4) specific to the biotinylated antigens immobilized on the sensing electrode.

Fig. 3C: shows placement of the sensing electrode in contact with a test solution containing DNA molecules (8) specific to the biotinylated DNA probes immobilized on the sensing electrode.

Fig. 4A: shows placement of the sensing electrode in contact with a solution of secondary receptors (9) conjugated with a charge label (10) and specific to the antigen under test (serological assay format, in which e.g. autoantibodies are determined by the use of anti-isotype antibodies conjugated with a charge label).

Fig. 4B: shows placement of the sensing electrode in contact with a solution of secondary receptors (11) conjugated with a charge label (10) and specific to

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the antibodies under test (sandwich assay format, in this case the secondary receptors are anti-isotype antibodies).

5 Fig. 5A: shows placement of the sensing electrode in contact with a solution of competing molecules (12) conjugated with a charge label (10) and specific to the biotinylated antibodies (competitive assay format).

10 Fig. 5B: shows placement of the sensing electrode in contact with a solution of competing molecules (13) conjugated with a charge label (10) and specific to the biotinylated antigens (competitive assay format).

15 Fig. 6A: shows placement of the sensing electrode in contact with a solution of secondary receptors (9) conjugated with an enzyme (14) and specific to the antigen under test (sandwich assay format).

20 Fig. 6B: shows placement of the sensing electrode in contact with a solution of secondary receptors (11) conjugated with an enzyme (14) and specific to the antibodies under test (serological assay format, in which e.g. autoantibodies are determined by the use 25 of anti-isotype antibodies conjugated with an enzyme).

30 Fig. 7A: shows placement of the sensing electrode in contact with a solution of competing molecules (12) conjugated with an enzyme (14) and specific to the biotinylated antibodies (competitive assay format).

35 Fig. 7B: shows placement of the sensing electrode in contact with a solution of competing molecules (13)

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conjugated with an enzyme (14) and specific to the biotinylated antigens (competitive assay format).

5 Fig. 8A: illustrates the steps of electrochemical analysis in a 'sequential' format with sequential placement of the sensing electrode in contact with a solution of biotinylated receptors [Fig. 8A1], with a test solution [Fig. 8A2], with a solution of labelled secondary receptors [Fig. 8A3], and subsequent 10 measurement of the sensing electrode potential relative to the potential of a reference electrode [Fig. 8A4].

15 Fig. 8B: illustrates the steps of electrochemical analysis in which the sensing electrode is first placed in contact with a solution of biotinylated receptors [Fig. 8B1], then the sensing electrode is placed in contact with a test solution to which a 20 solution of labelled secondary receptors has been added [Fig. 8B2], and the sensing electrode potential is then measured relative to the potential of a reference electrode [Fig. 8B3].

25 Fig. 8C: illustrates the steps of electrochemical analysis in a 'one-pot' format in which solutions of biotinylated receptors and labelled secondary receptors are added to a test solution [Fig. 8C1], then the sensing electrode is placed in contact with the test solution [Fig. 8C2], and the sensing 30 electrode potential is then measured relative to the potential of a reference electrode [Fig. 8C3].

35 Fig. 9: illustrates the typically shape of a curve of variation in sensing electrode potential for step-wise changes in the ionic strength or composition of

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the working solution (on the coordinates "millivolts -time") following incubation of the sensing electrode with a test solution not containing any analyte (curve 1), and following incubation of the sensing 5 electrode with a test solution containing analyte specific to the biotinylated receptors (curve 2).

Fig. 10: shows a calibration curve of the difference 10 in millivolts between the background and final potentials of the sensing electrode measured relative to the reference electrode versus HBsAg concentration in samples of blood serum.

Fig. 11: shows a curve of the difference in 15 millivolts between the background and final potentials of the sensing electrode measured relative to the reference electrode versus dilution of HBsAg-positive blood serum.

20 The reagents, materials and equipment used in the Examples were as follows:

Reagents and materials:

25 Pyrrole (> 98%) was purchased from Merck and before use was purified twice by vacuum distillation, then stored under N₂ in an opaque vessel at +4°C;

The following reagents were all purchased from Sigma Chemical Co. (USA):

30 Potassium hydroxide (KOH, ACS reagent), sodium hydroxide (NaOH, ACS reagent), sodium azide (SigmaUltra), ceric sulphate (ACS reagent), potassium chloride (SigmaUltra), sodium chloride (SigmaUltra), Tris[hydroxymethyl]aminomethane (SigmaUltra), sodium dodecylsulphate (SDS, >99%), sodium dextran sulphate

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(mol.wt. ~8000), isopropyl alcohol (ACS reagent), acetone, hydrochloric acid (ACS reagent), chloric acid (ACS reagent), N,N-dimethyl formamide (ACS reagent), glycine buffer solution 0.2M), "Tris buffered saline tablets", "Phosphate buffered saline tablets", o-Phenylenediamine dihydrochloride tablets", bovine serum albumin (RIA grade, fraction V), frozen bovine serum, streptavidin (from 5 *Streptomyces avidinii*), NHS-d-biotin, dimethyl sulfoxide (DMSO, ACS reagent), biotin-X-X-NHS, rabbit 10 polyclonal antibodies to bovine albumin, conjugate of goat polyclonal antibodies to mouse IgG with urease, digoxin, mouse monoclonal anti-digoxin clone DI-22 biotin conjugate, peroxidase (100 units per mg 15 solid), disposable dialysis bags (MWCO 1,000); Unless otherwise stated, phosphate buffered saline solution (PBS) was used throughout at pH 7.4.

20 Mouse monoclonal antibodies to Hepatitis B Surface Antigen (HBsAg) (5.7 mg/ml in phosphate buffered saline solution with 0.01% sodium azide), sheep polyclonal antibodies to mouse IgG (10.0 mg/ml in phosphate buffered saline solution with 0.01% sodium azide) and conjugate of mouse monoclonal antibodies 25 to HBsAg with peroxidase (2.5 mg/ml in phosphate buffered saline solution with 0.01% sodium azide) were purchased from Sorbent-Service Ltd. (Russia);

30 Lyophilised insulin (bovine, ~20IU/mg) and mouse monoclonal antibodies to insulin (1.2 mg/ml in phosphate buffered saline solution), kindly contributed by members of the Cardiology Research Centre attached to the Russian Academy of Medical Sciences;

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Preparation of lyophilised double-strand DNA-probe
(-1kb in length) biotinylated by nick-translation
[20], preparation of lyophilised double-strand DNA
complementary to biotinylated DNA-probe, preparation
5 of lyophilised double-strand DNA non-complementary to
biotinylated DNA-probe, preparation of lyophilised
DNA from salmon sperm and buffer solution for DNA
hybridisation, all kindly contributed by members of
the Scientific Research Institute of Biophysics
10 attached to the Ministry of Health.

Polybead® Sulphate Microspheres (2.5% Solids-Latex,
0.2 μ m) were purchased from Polysciences Inc.;

15 Lavsan™ film (~500 μ m thickness), purchased from
Vladimir Chemical Plant (Russia);

Chromium target dispersed in vacuum and photoresist
«FP-383», purchased from NIIPIK Institute (Russia);
20

Deionised water (reagent grade, resistance > 18 M Ω)
obtained using a Millipore Milli-RO/Milli-Q System;

Platinum wire, thickness ~ 0.5 mm;

25 Second British Working Standard for Hepatitis B
Surface Antigen (HBsAg concentration 0.50 iU/ml),
kindly contributed by the North London Blood
Transfusion Centre, UK;

30 Samples of HBsAg-positive human sera, kindly
contributed by the North London Blood Transfusion
Centre, UK.

- 40 -

Equipment:

UVN vacuum deposition unit (Russia);

«PNF-6Ts» photoresist application unit (Russia);

5 Self-produced photo-template (a photo-template which yields the electrode design shown in Figure 1, Figure 3 or Figure 4 of WO 96/02001 is suitable);

10 «STP-300» photo-exposure unit (Russia);

Self-made Soxhlet apparatus;

EU 18 dry heat cabinet (Jouan, France);

15 «PI-50.1» potentiostat-galvanostat with standard integrator and twin-coordinate recorder (Russia);

Two-channel recorder 2210 (LKB-Pribori, Sweden);

20 Checkmate pH meter with standard combination electrode (Mettler, Switzerland);

25 Self-produced Ag/AgCl semi-micro reference electrode, diameter ~ 2.5 mm and filled with saturated KCl solution;

Ag/AgCl reference electrode N° 476370 (Corning) ;

30 Purpose designed PC-compatible measuring instrument, including amplifier and analog-to-digital converter, controlled by custom software;

35 Purpose designed sensing electrode and reference electrode holder.

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Example 1.

HBsAg determination: competitive assay; sample-diluted specimens; receptor-biotinylated monoclonal mouse anti-HBsAg; competing molecule-labelled sheep polyclonal anti-mouse IgG; charge label-latex.

1.1. Electrode substrates were prepared from 60 x 48mm Lavsan film, then washed in hot isopropanol and dried in isopropanol vapour. A 0.05 μ m layer of chromium was applied to the substrates by magnetron deposition. Photoresist was applied to the chromium layer by centrifugation and dried at +80°C for 20 minutes. The photoresist layer was exposed using ultraviolet light through a patterned phototemplate. The photoresist layer was developed in KOH solution, then dried at +100°C for 20 minutes. The chromium pattern was obtained by etching in ceric sulphate solution. The photoresist was removed using dimethylformamide, followed by rinsing and drying in isopropanol vapour. A 0.50 μ m layer of gold was applied to the chromium pattern by galvanic deposition from auric chloride solution, followed by rinsing and drying in isopropanol vapour.

1.2. The electrodes thus obtained were washed twice in 2-5% KOH solution for 30 minutes, then rinsed with deionised water and washed twice in acetone for 5 minutes, then air dried at room temperature for 20 minutes. The electrodes were then mounted in a fluoroplastic holder and placed in a Soxhlet vessel where they were washed in hot isopropyl alcohol for 0.5-2 hours. The holder complete with electrodes was then removed from the Soxhlet vessel and the electrodes were dried in isopropyl alcohol vapour. On completion of these procedures, the electrodes were

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placed in a sealed glass vessel.

1.3. A storage solution for the sensing electrodes was made up by dissolving 8.0g of sodium chloride, 5 12.2g of Tris(hydroxymethyl)aminomethane, 0.2g of potassium chloride and 0.1g of sodium azide in 1 litre of deionised water.

1.4. Mouse monoclonal antibodies to HBsAg were 10 biotinylated, as follows :

0.01M tris-saline buffered solution (pH 8.0) containing sodium azide was made by dissolving 20 tris buffered saline tablets and 0.15g of sodium azide in 1.50 litres of deionised water;

15 1.0mg NHS-d-biotin was dissolved in 1ml of DMSO;

20 176 μ l of the purchased mouse monoclonal antibodies to HBsAg (5.7mg/ml) were added to a microtube containing 824 μ l of 0.01M phosphate buffered saline solution;

25 50 μ l of NHS-d-biotin solution in DMSO were added to the solution obtained;

the microtube containing the mixture was placed in a thermomixer and held for 4 hours at a temperature of +22°C with continuous mixing;

30 the mixture was then dialysed at +4°C overnight against a 500-fold excess of 0.01M tris-saline buffered solution containing sodium azide;

35 the resultant solution of biotinylated

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antibodies was divided into aliquots of small volume (~10 μ l) and stored at +4°C.

1.5. Sheep polyclonal antibodies to mouse IgG were
5 conjugated with latex microspheres, as follows :

10 a glycine buffered saline solution (pH 8.2) containing sodium azide was made up by adding 500ml of 0.2M glycine buffer solution to 500ml of deionised water, dissolving 8.5g of sodium chloride and 0.1g of sodium azide in the resultant mixture, and adjusting the pH to 8.2 with 0.1M NaOH solution;

15 60 μ l of the purchased sheep polyclonal antibodies to mouse IgG (10mg/ml) were added to a microtube containing 940 μ l of glycine buffered saline solution (preferably the polyclonal antibodies are affinity purified before
20 labelling);

25 200 μ l of a 2.5% suspension of Polybead® Sulphate Microspheres were added to the resultant solution;

30 the microtube containing the mixture was placed in a thermomixer and held for 30 minutes at a temperature of 37±1°C with continuous mixing;

35 the microtube containing the mixture was then cooled to room temperature and 0.01g of bovine serum albumin was added to it;

the resultant solution of labelled antibodies was divided into aliquots of small volume (~5 μ l)

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and stored at +4°C.

1.6. Prior to polymerisation, the monomer (e.g. 5
pyrrole) is distilled in standard water cooled
apparatus at atmospheric pressure at 135-140°C, and
stored in a sealed opaque vessel under N₂ at -20°C to
-5°C. The concentration of monomer in the
electrochemical polymerisation solution is varied
according to the type of test within the range 0.3 -
10 1.0 M.

In this example a solution was made up for
electrochemical polymerisation of pyrrole as follows:

15 2.5ml of freshly-distilled pyrrole and 0.02g of
SDS were dissolved in 20.0ml of deionised water;

20 a phosphate-saline buffer tablet was dissolved
in 200ml of deionised water and 4.0mg of
streptavidin were dissolved in 2ml of the PBS;

1ml of streptavidin solution in PBS was added to
the solution of pyrrole and SDS;

25 the final solution was placed in an orbital
mixer and mixed for 10 minutes.

1.7. The electrodeposition process is carried out in
a triple-electrode electrochemical cell, including a
30 working electrode, and reference electrode and an
auxiliary (counter) electrode. The working electrode
is a metal potentiometric electrode prepared as
described above, the auxiliary electrode is a length
of gold or platinum wire, and the reference is
35 silver/silver chloride electrode.

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Deposition is carried out using a potentiostat, applying a continuous voltage sweep on the working electrode. Depending on the desired thickness and properties of the polymer film, the lower potential sweep boundary, the upper potential sweep boundary, the voltage sweep rate and the number of sweep cycles are varied, typically from -500mV to +800mV, +1000mV to +2000mV (relative to the Ag/AgCl reference electrode), and 25 - 200 mV/sec. and 3-30 respectively.

In this example a polypyrrole film was formed by electrochemical deposition with binding of streptavidin on the electrode, as follows :

200 μ l of electrochemical polymerisation solution were placed in a well of a microtiter plate;

the electrode, platinum wire and semi-micro reference electrode, connected to the potentiostat, were immersed in the well;

a cyclical sweep of the electrode potential relative to the reference electrode was applied in the range +800 to +1800mV at a sweep rate of 150mV/sec.;

the process of formation of the polypyrrole film was monitored with reference to the volt-ampere curve using a twin-coordinate chart recorder connected to the corresponding outputs of the potentiostat, and with reference to the total quantity of electricity passing through the electrode using an integrator and chart recorder connected to the corresponding outputs of the

potentiostat. Throughout the deposition procedure checks are made to ensure that the quantity of electricity passing through the working electrode in the first and subsequent cycles does not differ by more than 15%;

10

on reaching the specified thickness of polypyrrole film (number of potential sweep cycles - 8; total quantity of electricity through the electrode ~ 750 mC), the process was stopped.

1.8. The sensing electrode coated in polypyrrole film with bound streptavidin was removed from the well, rinsed with deionised water followed by 0.01M phosphate-saline buffer solution (pH 7.4), and placed in a microtube with 300 μ l of storage solution, where it was stored at +4°C.

20

1.9. To obtain the required quantity of sensing electrodes steps 1.7-1.8 were repeated.

25

30

1.10. A series of 200 μ l samples of known HBsAg concentration were prepared in separate microtubes by diluting Second British Working Standard for Hepatitis B Surface Antigen in previously thawed bovine serum with a dilution factor of 2, 4, 8 and 10, adding 100, 150, 175 and 180 μ l of bovine serum to 100, 50, 25 and 20 μ l of Second British Working Standard for Hepatitis B Surface Antigen, respectively. Pure bovine serum was used to provide a sample with zero HBsAg content.

35

1.11. 0.2ml of a suitably titered solution of the biotinylated mouse monoclonal antibodies to HBsAg was

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added to 19.8ml of 0.01M phosphate buffered saline solution, thoroughly mixed in an orbital shaker, then dispensed into microtubes in aliquots of 200 μ l.

5 1.12. 0.1ml of a suitably titered solution of the labelled (latex conjugated) sheep polyclonal antibodies to mouse IgG was added to 19.9ml of 0.01M phosphate buffered saline solution, thoroughly mixed in an orbital mixer, then dispensed into microtubes 10 in aliquots of 200 μ l.

1.13. Working buffer solution N°1 was made up, as follows:

15 a phosphate-saline buffer tablet was dissolved in 200ml of deionised water;
20 2g of bovine serum albumin and 0.37g of KCl were dissolved in the solution obtained.

1.14. Working buffer solution N°2 was made up by dissolving a phosphate-saline buffer tablet in 200ml of deionised water.

25 1.15. The appropriate number of sensing electrodes coated in polypyrrole film with bound streptavidin were removed from the storage buffer solution and each placed in one of the microtubes containing the solution of biotinylated mouse 30 monoclonal antibodies to HBsAg (from step 1.11), and incubated for 10 minutes at room temperature.

1.16. On completion of 1.15, the sensing electrodes were removed from the microtubes containing biotinylated antibodies solution and each

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placed in one of the microtubes containing samples of known HBsAg concentration (from step 1.10); the microtubes with sensing electrodes were then placed in a thermomixer and held for 15 minutes at a 5 temperature of $37\pm1^{\circ}\text{C}$, mixing continuously.

1.17. On completion of 1.16, the sensing electrodes were removed from the microtubes containing the samples and placed in the microtubes 10 containing latex conjugated sheep polyclonal antibodies to mouse IgG (from step 1.12), then placed in a rotary shaker and held for 5 minutes at room temperature, mixing continuously.

1.18. On completion of 1.17, the sensing electrodes were removed from the microtubes, rinsed 15 for 3-5 seconds in 0.01M phosphate buffered saline solution, and each placed in a microtiter plate well filled with working buffer solution N°1.

1.19. The sensing electrode and reference electrode were connected to the electrical contacts of the holder connected to the PC-based measuring device, and the holder was positioned over the 20 microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and reference electrode were immersed in the solution.

1.20. The custom software was started and used to record the sensing electrode potential in millivolts 30 relative to the reference electrode potential for a period of 30 seconds.

1.21. On completion of 1.20, the holder was 35 positioned over a microtiter plate well filled with

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working buffer solution N°2, in a manner similar to that described in 1.19.

1.22. The custom software was used to record the 5 variation in millivolts of the sensing electrode potential relative to the reference electrode potential over a period of 100 seconds.

1.23. Using the custom software, the difference δ 10 in millivolts between the background and final potential of the sensing electrode was calculated.

1.24. The operations described in 1.19 - 1.21 were repeated in sequence using the samples of known 15 HBsAg concentration prepared during step 1.10.

1.25. Based on the results obtained during step 1.24, the custom software was used to plot the curve "δ against HBsAg concentration in the sample", (Fig. 20 10) and the lower threshold of absolute sensitivity of the sensing electrode system was determined from this curve.

25 Example 2.

HBsAg determination: competitive assay; sample-
diluted specimens; receptor-biotinylated monoclonal
mouse anti-HBsAg; competing molecule-labelled sheep
polyclonal anti-mouse IgG; charge label-latex.

30 2.1. The procedures described in 1.1 - 1.5 were carried out.

2.2. A solution was made up for electrochemical 35 polymerisation of pyrrole, as follows:

- 50 -

2.5ml of freshly-distilled pyrrole and 0.05g of SDS were dissolved in 20.0ml of deionised water;

2.3. A polypyrrole film was formed by electrochemical deposition, as follows :

5 200 μ l of electrochemical polymerisation solution were placed in a well of a microtiter plate;

10 the electrode, platinum wire and semi-micro reference electrode, connected to the potentiostat, were immersed in the well;

15 a cyclical sweep of the electrode potential relative to the reference electrode was applied in the range +800 to +2200mV at a sweep rate of 100mV/sec.;

20 the process of formation of the polypyrrole film was monitored with reference to the volt-ampere curve using an X-Y chart recorder connected to the corresponding outputs of the potentiostat, and with reference to the total quantity of electricity passing through the electrode using an integrator and chart recorder connected to the corresponding outputs of the potentiostat;

25 30 on reaching the specified thickness of polypyrrole film (number of potential sweep cycles - 6; total quantity of electricity through the electrode - 750 mC), the process was stopped.

35 2.4. The sensing electrode coated in polypyrrole film was removed from the well, rinsed with deionised

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water followed by 0.01M phosphate-saline buffer solution (pH 7.4), and placed in a microtube with 300 μ l of storage solution, where it was stored at +4°C.

5

2.5. To obtain the required quantity of sensing electrodes, the procedures described in 2.1 - 2.4 were repeated.

10

2.6. A solution of streptavidin was made up by dissolving a phosphate-saline buffer tablet in 200ml of deionised water, and dissolving 1.0mg of streptavidin in the resultant solution.

15

2.7. Streptavidin was bound on the surface of the polypyrrole film covering the sensing electrode, as follows :

20

the streptavidin solution was dispensed into microtubes in 200 μ l aliquots;

25

the sensing electrodes coated with polypyrrole film were removed from the storage buffer solution, each placed in a microtube with streptavidin solution and incubated for 18 hours at +4°C;

30

the sensing electrodes were removed from the microtubes containing streptavidin solution, washed with a 0.01M phosphate buffered saline solution and each placed in storage buffer solution, then stored at +4°C.

35

2.8. A series of samples with known HBsAg concentration were prepared as described in 1.10.

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2.9. 0.1ml of a suitably titered solution of the biotinylated mouse monoclonal antibodies to HBsAg was added to 19.9ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker, then 5 dispensed into microtubes in aliquots of 200 μ l.

2.10. 0.1ml of a suitably titered solution of the labelled (latex conjugated) sheep polyclonal antibodies to mouse IgG was added to 19.9ml of 0.01M 10 phosphate buffered saline solution, thoroughly mixed in an orbital mixer, then dispensed into microtubes in aliquots of 200 μ l.

2.11. Working buffer solution N°1 was made up as 15 described in 1.13.

2.12. Working buffer solution N°2 was made up as described in 1.14.

2.13. The sensing electrodes coated in 20 polypyrrole film with bound streptavidin were removed from the storage buffer solution and each placed in a microtube containing the solution of biotinylated mouse monoclonal antibodies to HBsAg (from step 2.9), and incubated for 10 minutes at room temperature. 25

2.14. On completion of 2.13, the sensing electrodes were removed from the microtubes containing biotinylated antibodies solution and each 30 placed in one of the microtubes containing samples of known HBsAg concentration (from step 2.8); the microtubes with sensing electrodes were then placed in a thermomixer and held for 15 minutes at a temperature of 37±1°C, mixing continuously.

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2.15. On completion of 2.14, the sensing electrodes were removed from the microtubes containing the samples and placed in the microtubes containing the solution of latex conjugated sheep polyclonal antibodies to mouse IgG (from step 2.10), then placed in a rotary shaker and held for 5 minutes at room temperature, mixing continuously.

2.16. On completion of 2.15, the sensing electrodes were removed from the microtubes, rinsed for 3-5 seconds in 0.01M phosphate buffered saline solution, and each placed in a microtiter plate well filled with working buffer solution N°1.

2.17. The sensing electrode and reference electrode were connected to the electrical contacts of the holder connected to the PC-based measuring instrument, and the holder was positioned over the microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and reference electrode were immersed in the solution.

2.18. The custom software was started and used to record the sensing electrode potential in millivolts relative to the reference electrode potential for a period of 30 seconds.

2.19. On completion of 2.18, the holder was positioned over a microtiter plate well filled with working buffer solution N°2, in a manner similar to that described in 2.17.

2.20. The custom software was used to record the variation in millivolts of the sensing electrode potential relative to the reference electrode

- 54 -

potential over a period of 100 seconds.

2.21. Using the custom software, the difference
(δ) in millivolts between the background and final
5 potential of the sensing electrode was calculated.

2.22. The operations described in 2.17 - 2.20
were repeated in sequence using the samples of known
HBsAg concentration prepared at step 2.8.

10 2.23. Based on the results obtained at step 2.22,
the custom software was used to plot the calibration
curve " δ against HBsAg concentration in the sample".

15 2.24. A number of samples of HBsAg-positive blood
serum (containing an unknown concentration of HBsAg)
were each serially diluted in previously thawed
bovine serum to prepare a series of samples with
dilution factors of 10, 100, 1000, 5000 and 10 000. A
20 200 μ l aliquot of each dilution was placed in a
separate microtube.

2.25. The procedure described in steps 2.13-2.21
was used to determine the concentration of HBsAg in
25 each of the dilutions and these results, together
with the calibration curve obtained in step 2.23 were
used to calculate the concentration of HBsAg in the
original (undiluted) samples of blood serum (Fig.
11).

30

Example 3.

Anti-albumin antibody determination: competitive

assay; sample-rabbit polyclonal antibodies against

35 albumin; receptor-biotinylated bovine serum albumin;

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competing molecule-labelled rabbit polyclonal antibodies to albumin; charge label-latex.

3.1. The procedures described in 1.1 - 1.3 were
5 carried out.

3.2. Bovine serum albumin (BSA) was biotinylated as described in 1.4. The resultant solution of biotinylated BSA was divided into aliquots of small
10 volume (~10 μ l) and stored at +4°C.

3.3. Rabbit polyclonal antibodies to bovine albumin were conjugated with latex microspheres following the procedure described in 1.5. The resultant solution of
15 labelled antibodies was divided into aliquots of small volume (~5 μ l) and stored at +4°C.

3.4. A solution was made up for electrochemical polymerisation of pyrrole, as described in 2.2.

20 3.5. A polypyrrole film was formed by electrochemical deposition, as described in 2.3.

3.6. The sensing electrode coated in polypyrrole film
25 was removed from the well, rinsed with deionised water followed by 0.01M phosphate-saline buffer solution (pH 7.4), and placed in a microtube with 300 μ l of storage solution, where it was stored at +4°C.

30 3.7. To obtain the required quantity of sensing electrodes, the procedures described in 3.1 - 3.6 were repeated.

35 3.8. A solution of streptavidin was made up as

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described in 2.6.

3.9. Streptavidin was bound on the surface of the polypyrrole film covering the sensing electrode, as
5 described in 2.7.

3.10. A series of samples with known concentration of unlabelled rabbit polyclonal antibodies to bovine albumin were prepared, as
10 follows:

the purchased sample of rabbit polyclonal antibodies to bovine albumin was dialysed at +4°C overnight against a 500-fold excess of 0.01M phosphate buffered saline solution;

the resultant solution of rabbit polyclonal antibodies to bovine albumin in 0.01M phosphate buffered saline solution was serially diluted with 0.01M phosphate buffered saline solution at dilution factors of 10, 20, 50, 100, 1000 and 5000;

25 200µl aliquots of each of the diluted samples were placed in separate microtubes.

3.11. 0.8ml of a suitably diluted solution of biotinylated BSA was added to 19.2ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker, then dispensed into microtubes in aliquots of 200µl.

3.12. 0.1ml of a suitably titered solution of labelled (latex conjugated, as described above) rabbit polyclonal antibodies to bovine albumin was

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added to 19.9ml of 0.01M phosphate buffered saline solution, thoroughly mixed in an orbital mixer, then dispensed into microtubes in aliquots of 200 μ l.

5 3.13. Working buffer solution N°1 was made up, as follows:

a phosphate-saline buffer tablet was dissolved in 200ml of deionised water;

10 0.37g of KCl were dissolved in the solution obtained.

15 3.14. Working buffer solution N°2 was made up as described in 1.14.

20 3.15. The sensing electrodes coated in polypyrrole film with bound streptavidin were removed from the storage buffer solution and each placed in one of the microtubes containing 200 μ l of biotinylated BSA solution (from step 3.11), and incubated for 25 minutes at room temperature.

25 3.16. On completion of 3.15, the sensing electrodes were removed from the microtubes containing biotinylated BSA solution and each placed in one of the microtubes containing samples with known concentration of unlabelled rabbit polyclonal antibodies to bovine albumin (from step 3.10); the 30 microtubes with sensing electrodes were then placed in a thermomixer and held for 25 minutes at a temperature of 37±1°C, mixing continuously.

35 3.17. On completion of 3.16, the sensing electrodes were removed from the microtubes

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containing the samples and transferred to microtubes containing the solution of latex conjugated rabbit polyclonal antibodies to bovine albumin (from step 3.12), then placed in a rotary shaker and held for 10 minutes at room temperature, mixing continuously.

5 3.18. On completion of 3.17, the sensing electrodes were removed from the microtubes, rinsed for 3-5 seconds in 0.01M phosphate buffered saline 10 solution, and each placed in a microtiter plate well filled with working buffer solution N°1.

15 3.19. The sensing electrode and reference electrode were connected to the electrical contacts of the holder connected to the PC-based measuring instrument, and the holder was positioned over the microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and reference electrode were immersed in the solution.

20 3.20. The custom software was started and used to record the sensing electrode potential in millivolts relative to the reference electrode potential over a period of 100 seconds.

25 3.21. On completion of 3.20, the holder was positioned over a microtiter plate well filled with working buffer solution N°2, in a manner similar to that described in 3.19.

30 3.22. The custom software was used to record the variation in millivolts of the sensing electrode potential relative to the reference electrode potential over a period of 200 seconds.

- 59 -

3.23. Using the custom software, the area (integral) S_2 described by the curve of sensing electrode potential variation versus reference electrode potential was calculated.

5

3.24. The operations described in 3.19 - 3.23 were repeated in sequence using the samples with known concentration of unlabelled rabbit polyclonal antibodies to bovine albumin prepared at step 3.10.

10

3.25. Based on the results obtained at step 3.24, the custom software was used to plot the calibration curve " S_2 against concentration of unlabelled rabbit polyclonal antibodies to bovine albumin in the sample".

Example 4.

HBsAg determination; sandwich assay; sample-sample with known HBsAg concentration; receptor-biotinylated mouse monoclonal antibodies to HBsAg; labelled mouse monoclonal antibodies to HBsAg; label-peroxidase.

4.1. The procedures described in 1.1 - 1.4 were carried out.

4.2. A solution was made up for electrochemical polymerisation of pyrrole, as described in 2.2.

4.3. A polypyrrole film was formed by electrochemical deposition, as described in 2.3.

4.4. The sensing electrode coated in polypyrrole film was removed from the well, rinsed with deionised water followed by 0.01M phosphate-saline buffer

- 60 -

solution (pH 7.4), and placed in a microtube with 300 μ l of storage solution, where it was stored at +4°C.

5 4.5. To obtain the required quantity of sensing electrodes, the procedures described in 4.1-4.4 were repeated.

10 4.6. A solution of streptavidin was made up as described in 2.6.

4.7. Streptavidin was bound on the surface of the polypyrrole film covering the sensing electrode, as described in 2.7.

15 4.8. A series of samples with known HBsAg concentration were prepared, as described in 1.10.

20 4.9. 0.2ml of a suitably titered solution of the biotinylated mouse monoclonal antibodies to HBsAg was added to 19.8ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker, then dispensed into microtubes in aliquots of 200 μ l.

25 4.10. 5ml of previously thawed bovine serum were added to 15ml of 0.01M phosphate buffered saline solution, and 0.4ml of a suitably titered solution of purchased peroxidise conjugated mouse monoclonal antibodies to HBsAg was added to 19.6ml of the resultant solution, which was then thoroughly mixed in a rotary shaker and dispensed into microtubes in aliquots of 200 μ l.

30 4.11. Working buffer solution N°1 was made up by dissolving a phosphate-saline buffer tablet in 200ml

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of deionised water.

4.12. Working buffer solution N°2 was made up, as follows:

5

an o-Phenylenediamine dihydrochloride tablet and a urea hydrogen peroxide/buffer tablet were dissolved in 20ml of deionised water;

10

0.1ml of the solution obtained was added to 19.9ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker, then placed in an opaque glass vessel and stored at +4°C until the start of the test.

15

4.13. The sensing electrodes coated in polypyrrole film with bound streptavidin were removed from the storage buffer solution and each placed in a microtube containing 200µl of the solution of biotinylated mouse monoclonal antibodies to HBsAg (from step 4.9) and incubated for 5 minutes at room temperature.

20

4.14. On completion of 4.13, the sensing electrodes were removed from the microtubes containing biotinylated antibodies solution and placed in microtubes containing samples with known HBsAg concentration (from step 4.8); the microtubes with sensing electrodes were then placed in a thermomixer and held for 10 minutes at a temperature of 37±1°C, mixing continuously.

25

4.15. On completion of 4.14, the sensing electrodes were removed from the microtubes containing the samples and placed in microtubes

30

35

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containing the solution of peroxidase-conjugated mouse monoclonal antibodies to HBsAg (from step 4.10), then placed in a rotary shaker and held for 5 minutes at room temperature, mixing continuously.

5

4.16. On completion of 4.15, the sensing electrodes were removed from the microtubes, rinsed for 3-5 seconds in 0.01M phosphate buffered saline solution, and each placed in a microtiter plate well filled with working buffer solution N°1.

10

4.17. The sensing electrode and reference electrode were connected to the electrical contacts of the holder connected to the PC-based measuring instrument, and the holder was positioned over the microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and reference electrode were immersed in the solution.

20

4.18. The custom software was started and used to record the sensing electrode potential in millivolts relative to the reference electrode potential over a period of 50 seconds.

25

4.19. On completion of 4.18, the holder was positioned over a microtiter plate well filled with working buffer solution N°2, in a manner similar to that described in 4.17.

30

4.20. The custom software was used to record the variation in millivolts of the sensing electrode potential relative to the reference electrode potential over a period of 100 seconds.

35

4.21. Using the custom software, the difference

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(δ) in millivolts between the background and final potential of the sensing electrode was calculated.

4.22. The operations described in 4.17 - 4.21
5 were repeated in sequence using the samples of known
HBsAg concentration prepared at step 4.8.

4.23. Based on the results obtained at step 4.22,
the custom software was used to plot the calibration
10 curve " δ against HBsAg concentration in the sample".

4.24. A series of diluted blood serum samples
were prepared as described in 2.24.

15 4.25. The procedure described in steps 4.13 -
4.21 was used to determine the concentration of HBsAg
in each of the diluted samples prepared in 4.24 and
these results, together with the calibration curve
obtained in step 4.23 were used to calculate the
20 concentration of HBsAg in the original (undiluted)
samples of blood serum.

Example 5.

25 HBsAg determination; sandwich assay; sample-sample
with known HBsAg concentration; receptor-biotinylated
mouse monoclonal antibodies to HBsAg; labelled mouse
monoclonal antibodies to HBsAg; label-peroxidase;
"sequential assay".

30 5.1. The procedures described in 1.1 - 1.4 were
carried out.

35 5.2. A solution was made up for electrochemical
polymerisation of pyrrole, as described in 2.2.

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5.3. A polypyrrole film was formed by electrochemical deposition, as described in 2.3.

5.4. The sensing electrode coated in polypyrrole film was removed from the well, rinsed with deionised water followed by 0.01M phosphate-saline buffer solution (pH 7.4), and placed in a microtube with 300 μ l of storage solution, where it was stored at +4°C.

10 5.5. To obtain the required quantity of sensing electrodes, the procedures described in 5.1- 5.4 were repeated.

15 5.6. A solution of streptavidin was made up as described in 2.6.

20 5.7. Streptavidin was bound on the surface of the polypyrrole film covering the sensing electrode, as described in 2.7.

5.8. A series of samples with known HBsAg concentration were prepared, as described in 1.10.

25 5.9. 0.5ml of a solution of biotinylated mouse monoclonal antibodies to HBsAg was added to 19.5ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker, then dispensed into microtubes in aliquots of 200 μ l.

30 5.10. 1.7ml of a solution of peroxidase-labelled mouse monoclonal antibodies to HBsAg was added to 18.3ml of 0.01M phosphate buffered saline solution, thoroughly mixed in an orbital mixer and dispensed 35 into microtubes in aliquots of 200 μ l.

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5.11. Working buffer solution N°1 was made up as described in 4.11.

5.12. Working buffer solution N°2 was made up as 5 described in 4.12.

5.13. The sensing electrodes coated in polypyrrole film with bound streptavidin were removed from the storage buffer solution and each placed in a 10 microtube containing the solution of biotinylated mouse monoclonal antibodies to HBsAg (from step 5.9), and incubated for 5 minutes at room temperature.

5.14. Simultaneously with step 5.13, 10 μ l of a 15 suitably titered solution of the purchased peroxidase-labelled mouse monoclonal antibodies to HBsAg were added to each sample with known HBsAg concentration (from step 5.8), having first removed 10 μ l from the sample; the microtubes and samples were 20 then placed in a thermomixer at 37±1°C for 5-10 minutes.

5.15. On completion of 5.13 - 5.14, the sensing 25 electrodes were removed from the microtubes containing the solution of biotinylated antibodies and placed in the microtubes containing samples with known HBsAg concentration (from step 5.14) then held in the thermomixer for 15 minutes at 37±1°C, mixing continuously.

30 5.16. On completion of 5.15, the sensing electrodes were removed from the microtubes, rinsed for 3-5 seconds in 0.01M phosphate buffered saline solution, and each placed in a microtiter plate well 35 filled with working buffer solution N°1.

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5.17. The sensing electrode and reference electrode were connected to the electrical contacts of the holder connected to the PC-based measuring instrument, and the holder was positioned over the 5 microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and reference electrode were immersed in the solution.

5.18. The custom software was started and used to record the sensing electrode potential in millivolts relative to the reference electrode potential over a 10 period of 50 seconds.

5.19. On completion of 5.18, the holder was positioned over a microtiter plate well filled with 15 working buffer solution N°2, in a manner similar to that described in 5.17.

5.20. The custom software was used to record the 20 variation in millivolts of the sensing electrode potential relative to the reference electrode potential over a period of 200 seconds.

5.21. Using the custom software, the area 25 (integral) S_2 described by the curve of sensing electrode potential variation versus reference electrode potential was calculated.

5.22. The operations described in 5.17 - 5.21 30 were repeated in sequence using the samples with known HBsAg concentration prepared at step 5.8.

5.23. Based on the results obtained at step 5.22, 35 the custom software was used to plot the calibration curve " S_2 against HBsAg concentration in the sample".

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5.24. A series of diluted blood serum samples were prepared as described in 2.24.

5.25. The procedure described in steps 5.13 - 5.21 was used to determine the concentration of HBsAg in each of the diluted samples prepared in 5.24 and these results, together with the calibration curve obtained in step 5.23, were used to calculate the concentration of HBsAg in the original (undiluted) 10 samples of blood serum.

Example 6.

HBsAg determination; sandwich assay; sample-sample with known HBsAg concentration; receptor-biotinylated mouse monoclonal antibodies to HBsAg; labelled mouse monoclonal antibodies to HBsAg; label-peroxidase; "one-pot assay".

6.1 The procedures described in 1.1 - 1.4 were carried out.

6.2. A solution was made up for electrochemical polymerisation of pyrrole, as described in 2.2.

6.3. A polypyrrole film was formed by electrochemical deposition, as described in 2.3.

6.4. The sensing electrode coated in polypyrrole film was removed from the well, rinsed with deionised water followed by 0.01M phosphate-saline buffer solution (pH 7.4), and placed in a microtube with 300 μ l of storage solution, where it was stored at +4 °C.

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6.5. To obtain the required quantity of sensing electrodes, the procedures described in 6.1 - 6.4 were repeated.

5 6.6. A solution of streptavidin was made up as described in 2.6.

6.7. Streptavidin was bound on the surface of the polypyrrole film covering the sensing electrode, as 10 described in 2.7.

6.8. A series of samples with known HBsAg concentration were prepared, as described in 1.10.

15 6.9. 2.5ml of a suitably titered solution of the biotinylated mouse monoclonal antibodies to HBsAg was added to 17.5ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker, then dispensed into microtubes in aliquots of 200 μ l.

20 6.10. 1.7ml of a suitably titered solution of the purchased peroxidase-labelled mouse monoclonal antibodies to HBsAg was added to 18.3ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker and dispensed into microtubes in 25 aliquots of 200 μ l.

6.11. Working buffer solution N°1 was made up as described in 4.11.

30 6.12. Working buffer solution N°2 was made up as described in 4.12.

35 6.13. 10 μ l of a solution of biotinylated mouse monoclonal antibodies to HBsAg. (from step 6.9) and

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10 μ l of peroxidase-labelled mouse monoclonal antibodies to HBsAg (from step 6.10) were added to each sample of known HBsAg concentration, having first removed 20 μ l from the sample; the microtubes 5 and samples were then placed in a thermomixer, where they were incubated for 10 minutes at 37 \pm 1°C, mixing continuously.

6.14. On completion of 6.13, the sensing 10 electrodes coated in polypyrrole film with bound streptavidin were removed from the storage buffer solution, each placed in the microtubes containing samples with known HBsAg concentration (from step 6.13), then held in the thermomixer for 5 minutes at 15 a temperature of 37 \pm 1°C, mixing continuously.

6.15. On completion of 6.14, the sensing 20 electrodes were removed from the microtubes, rinsed for 3-5 seconds in 0.01M phosphate buffered saline solution, and each placed in a microtiter plate well filled with working buffer solution N°1.

6.16. The sensing electrode and reference 25 electrode were connected to the electrical contacts of the holder connected to the PC-based measuring instrument, and the holder was positioned over the microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and reference electrode were immersed in the solution.

30 6.17. The custom software was started and used to record the sensing electrode potential in millivolts relative to the reference electrode potential over a period of 100 seconds.

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6.18. On completion of 6.17, the holder was positioned over a microtiter plate well filled with working buffer solution N°2, in a manner similar to that described in 6.16.

5

6.19. The custom software was used to record the variation in millivolts of the sensing electrode potential relative to the reference electrode potential over a period of 200 seconds.

10

6.20. Using the custom software, the area (integral) S_2 described by the curve of sensing electrode potential variation versus reference electrode potential was calculated.

15

6.21. The operations described in 6.16 - 6.20 were repeated in sequence using the samples with known HBsAg concentration prepared at step 6.8.

20

6.22. Based on the results obtained at step 6.21, the custom software was used to plot the calibration curve " S_2 - HBsAg concentration in the sample".

25

6.23. A series of diluted blood serum samples were prepared as described in 2.24.

30

6.24. The procedure described in steps 6.13 - 6.21 was used to determine the concentration of HBsAg in each of the diluted samples prepared in 6.23 and these results, together with the calibration curve obtained in step 6.22, were used to calculate the concentration of HBsAg in the original (undiluted) samples of blood serum.

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Example 7.

Insulin determination; competitive assay; sample-samples with known insulin conc; receptor-biotinylated mouse monoclonal antibodies to insulin; 5 competing molecule-labelled polyclonal goat anti-mouse IgG antibodies; label-urease

7.1. The procedures described in 1.1 - 1.3 were carried out.

10

7.2. Biotinylation of mouse monoclonal antibodies to insulin was carried out as described in 1.4. The resultant solution of biotinylated mouse monoclonal antibodies to insulin was divided into aliquots of 15 small volume (~10 μ l) and stored at +4°C.

7.3. A solution was made up for electrochemical polymerisation of pyrrole, as described in 2.2.

20

7.4. A polypyrrole film was formed by electrochemical deposition, as described in 2.3.

25

7.5. The sensing electrode coated in polypyrrole film was removed from the well, rinsed with deionised water followed by 0.01M phosphate-saline buffer solution (pH 7.4), and placed in a microtube with 300 μ l of storage solution, where it was stored at +4°C.

30

7.6. To obtain the required quantity of sensing electrodes, the procedures described in 7.4-7.5 were repeated.

35

7.7. A solution of streptavidin was made up as described in 2.6.

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7.8. Streptavidin was bound on the surface of the polypyrrole film covering the sensing electrode, as described in 2.7.

5 7.9. A series of samples with known insulin concentration were prepared, as follows:

10 1.12g of potassium chloride and 1.0g of bovine serum albumin were dissolved in 100ml of deionised water;

15 100 μ g of lyophilised insulin were dissolved in 200 μ l of the solution obtained;

20 15 the insulin solution obtained was sequentially diluted with deionised water with potassium chloride and bovine serum albumin at dilution factors of 10, 20, 50, 100, 1000, 5000 and 10,000 times;

25 20 200 μ l aliquots of each of the diluted samples were placed in separate microtubes.

25 7.10. 0.8ml of a suitably titered solution of the biotinylated mouse monoclonal antibodies to insulin were added to 19.2ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker, then aliquotted into microtubes in quantities of 200 μ l.

30 7.11. 0.02 ml of purchased urease conjugated goat polyclonal antibodies to mouse IgG was added to 19.98ml of 0.01M phosphate buffered saline solution, thoroughly mixed in a rotary shaker and aliquotted 35 into microtubes in quantities of 200 μ l.

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7.12. Working buffer solution N°1 was made up by dissolving 2.24g of potassium chloride in 200ml of deionised water.

5 7.13. Working buffer solution N°2 was made up by dissolving 0.012g of urea in 20ml of working buffer solution N°1.

10 7.14. The sensing electrodes coated in polypyrrole film with bound streptavidin were removed from the storage solution, each placed in a microtube with the solution of biotinylated mouse monoclonal antibodies to insulin (from step 7.10), and incubated for 10 minutes.

15 7.15. On completion of 7.14, the sensing electrodes were removed from the microtubes with the solution of biotinylated antibodies and placed in microtubes containing samples of known insulin 20 concentration (from step 7.9), then placed in a thermomixer and held for 15 minutes at a temperature of $37\pm1^{\circ}\text{C}$, mixing continuously.

25 7.16. On completion of 7.15, the sensing electrodes were removed from the microtubes containing the samples and placed in microtubes containing the solution of urease conjugated goat polyclonal antibodies to mouse IgG (from step 7.11), then placed in a rotary shaker and held for 10 30 minutes at room temperature, mixing continuously.

35 7.17. On completion of 7.16, the sensing electrodes were removed from the microtubes, rinsed for 3-5 seconds in deionised water with potassium chloride and bovine serum albumin, and each placed in

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microtiter plate well filled with working buffer N°1.

7.18. The sensing electrode and reference electrode were connected to the electrical contacts of the holder connected to the PC-based measuring device, and the holder was positioned over the microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and reference electrode were immersed in the solution.

10 7.19. The custom software was started and used to record the sensing electrode potential in millivolts relative to the reference electrode potential over a period of 200 seconds.

15 7.20. On completion of 7.19, the holder was positioned over a microtiter plate well filled with working buffer solution N°2, in a manner similar to that described in 7.18.

20 7.21. The custom software was used to record the variation in millivolts of the sensing electrode potential relative to the reference electrode potential over a period of 400 seconds.

25 7.22. Using the custom software, the area (integral) S, described by the curve of sensing electrode potential variation versus reference electrode potential was calculated.

30 7.23. The operations described in 7.18 - 7.22 were repeated in sequence using the samples with known insulin concentration prepared at step 7.9.

35 7.24. Based on the results obtained at step 7.23,

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the custom software was used to plot the calibration curve "S₂ against insulin concentration in the sample".

5

Example 8.

Nucleic acid hybridisation.

8.1. The procedures described in 1.1 - 1.3 were
10 carried out.

8.2. A solution was made up for electrochemical polymerisation of pyrrole, as described in 2.2.

15 8.3 A polypyrrole film was formed by electrochemical deposition, as described in 2.3.

8.4. The sensing electrode coated in polypyrrole film
was removed from the well, rinsed with deionised
20 water followed by 0.01 M phosphate saline buffer
solution (pH 7.4), and placed in a microtube with
300 μ l of storage solution, where it was stored at
+4°C.

25 8.5. To obtain the required quantity of sensing
electrodes, the procedures described in 8.3 - 8.4
were repeated.

30 8.6. A solution of streptavidin was made up by
dissolving a phosphate buffered saline tablet in
200ml of deionised water and dissolving 5.0mg of
streptavidin in the solution obtained.

35 8.7. Streptavidin was bound on the surface of the
polypyrrole film covering the sensing electrode, as

follows:

the streptavidin solution was dispensed into microtubes in aliquots of 300 μ l;

5

the sensing electrodes coated with polypyrrole film were removed from the storage solution, each placed in a microtube containing streptavidin solution and incubated for 24 hours

10

at +4°C;

15

the sensing electrodes were removed from the microtubes containing streptavidin solution, rinsed with 0.01M phosphate buffered saline solution and twice with deionised water containing 0.01% sodium azide and 0.15M potassium chloride: each sensing electrode was then placed in storage solution and stored at +4°C.

20

8.8 A solution of biotinylated single-strand DNA probe was prepared, as follows:

25

1mg of lyophilised preparation of biotinylated double strand DNA probe (~1kb in length) was dissolved in 1ml of deionised water, and the solution obtained was placed in a microtube.

30

the microtube containing the DNA probe solution was placed in a water bath where it was incubated for 5-8 minutes at +100°C;

35

the microtube containing the DNA probe was transferred to a vessel containing ice, where it was rapidly cooled to 0°C;

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the microtube containing the DNA probe was then transferred to a freezer where it was stored frozen at -20°C.

5 8.9. A solution of single-strand DNA complementary to biotinylated DNA-probe was prepared, as follows:

10 10mg of lyophilised preparation of double-strand DNA complementary to biotinylated DNA-probe was dissolved in 1ml of deionised water, and the solution obtained was placed in a microtube;

15 the microtube containing the DNA solution was placed in a water bath, where it was incubated for 5-8 minutes at +100°C;

20 the microtube containing the DNA solution was transferred to a vessel containing ice, where it was rapidly cooled to 0°C;

25 the microtube containing the DNA solution was then transferred to a freezer where it was stored frozen at -20°C.

25 8.10. A solution of single-strand DNA non-complementary to biotinylated DNA probe was prepared as described in 8.9.

30 8.11 Working buffer solution N°1 was made up as follows:

a phosphate buffered saline tablet was dissolved in 200ml of deionised water;

35 2g of bovine serum albumin, 0.37g of potassium

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chloride and 0.12g of sodium citrate were dissolved in the solution obtained.

8.12. Working buffer solution N°2 was made up as
5 follows:

a phosphate buffered saline tablet was dissolved in 200ml of deionised water;

10 2g of bovine serum albumin and 1g of sodium dextran sulphate were dissolved in the solution obtained.

8.13. The sensing electrodes coated in
15 polypyrrole film with bound streptavidin were removed from the deionised water containing 0.01% sodium azide and 0.15M potassium chloride, and 20 μ l of single-strand DNA probe solution previously thawed and warmed to room temperature was applied to working
20 the surface of each sensing electrode.

8.14. On completion of 8.13, the sensing electrodes were placed in a humidity chamber where they were incubated for 60 minutes at +44°C.

25 8.15. On completion of 8.14, the sensing electrodes were removed from the humidity chamber and each placed in a microtube containing 200 μ l of initial buffer solution for DNA hybridisation, where
30 they were held for a short period of time at +4°C.
(The DNA hybridisation buffer may be any standard hybridisation buffer known in the art, see Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual, 2nd Edition*, Cold
35 Spring Harbor Laboratory Press, Cold Spring Harbor,

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NY, USA)

8.16. Samples containing single-strand DNA complementary to biotinylated DNA probe were
5 prepared, as follows:

10mg of a preparation of lyophilised DNA from salmon sperm was dissolved in 10ml of initial buffer solution for DNA hybridisation.

10 10 μ l of the solution of single-strand DNA complementary to biotinylated DNA-probe previously thawed and warmed to room temperature were added to 0.99ml of the solution obtained:

15 the resulting solution was thoroughly mixed in a rotary shaker and dispensed into microtubes aliquots of 200 μ l.

20 8.17. Samples containing single-strand DNA non-complementary to biotinylated DNA probe were prepared, as follows:

25 10mg of a preparation of lyophilised DNA from salmon sperm was dissolved in 10ml of initial buffer solution for DNA hybridisation;

30 100 μ l of the solution of single-strand DNA non-complementary to biotinylated DNA probe previously thawed and warmed to room temperature were added to 0.9ml of the solution obtained;

35 the resulting solution was thoroughly mixed in a rotary shaker and dispensed into microtubes in

- 80 -

aliquots of 200 μ l.

8.18. Half of the sensing electrodes with immobilised biotinylated single-strand DNA-probe were removed from the microtubes containing initial buffer solution for DNA hybridisation and placed in the microtubes with samples containing DNA complementary to biotinylated DNA-probe; the microtubes containing the sensing electrodes were then placed in a thermomixer and held for 120 minutes at +42°C mixing continuously.

8.19. On completion of 8.18, the sensing electrodes were removed from the microtubes, rinsed for 3-5 seconds in working buffer solution N°1 and each placed in the well of a microtiter plate filled with working buffer solution N°1.

8.20. The sensing electrode and reference electrode were connected to the electrical contacts of the holder connected to the PC-based measuring device, and the holder was positioned over the microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and reference electrode were immersed in the solution.

8.21. The custom software was started and used to record the sensing electrode potential in millivolts relative to the reference electrode potential over a period of 200 seconds.

8.22. On completion of 8.21, the holder was positioned over a microtiter plate well filled with working buffer solution N°2, in a manner similar to that described in 8.20.

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8.23. The custom software was used to record the variation in millivolts of the sensing electrode potential relative to the reference electrode potential over a period of 600 seconds.

5

8.24. Using the custom software, the difference (δ) in millivolts between the background and final potential values was calculated.

10

8.25. The operations described in 8.20 - 8.24 were repeated in sequence using the samples containing DNA complementary to biotinylated DNA probe prepared at step 4.8.

15

8.26. The other half of the sensing electrodes with immobilised biotinylated single-strand DNA probe were removed from the microtubes containing initial buffer solution for DNA hybridisation and placed in the microtubes with samples containing DNA non-complementary to DNA probe, the microtubes containing the sensing electrode were then placed in a thermomixer and held for 120 minutes at +42°C, mixing continuously.

20

25

8.27. The procedures described in 8.19 - 8.25 were repeated using the samples containing DNA non-complementary to biotinylated DNA probe.

30

8.28. Based on the results obtained in 8.19 - 8.26, the custom software was used to plot statistical distribution curves of the δ values obtained with the samples of DNA complementary and non-complementary to biotinylated DNA probe.

35

Example 9.

Digoxin determination; competitive assay; samples-
samples with known digoxin concentration; receptor-
biotinylated mouse monoclonal antibodies to digoxin;
5 competing molecule-labelled digoxin; label-
peroxidase.

9.1 The procedures described in 1.1-1.4 were carried
out.

10 9.2 A solution was made up for electrochemical
polymerisation of pyrrole, as described in 2.2.

15 9.3 A polypyrrole film was formed by electrochemical
deposition, as described in 2.3.

20 9.4 The sensing electrode coated in polypyrrole film
was removed from the well, rinsed with deionised
water followed by 0.01M phosphate-saline buffer
solution (pH 7.4) and placed in a microtube with
300 μ l of storage solution, where it was stored at
+4°C.

25 9.5 To obtain the required number of sensing
electrodes, the procedures described in 9.1-9.4 were
repeated.

9.6 A solution of streptavidin was made up as
described in 2.6.

30 9.7 Streptavidin was bound on the surface of the
polypyrrole film covering the sensing electrode, as
described in 2.7.

35 9.8 A series of samples with known digoxin

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concentration were prepared as follows:

250ml of ethanol were added to 750ml of deionised water;

5

250mg of digoxin were dissolved in 1000ml of the ethanol solution obtained;

10

a phosphate saline buffer tablet and 10.0g of bovine serum albumin were dissolved in 200ml of deionised water;

15

the digoxin solution obtained was sequentially diluted in PBS solution with bovine serum albumin at dilution rates of 250, 2500, 25000, 50000, 125000, 250000 and 500000 times;

20

200 μ l aliquots of each of the diluted samples were placed in separate microtubes.

25

9.9 20 μ l of the purchased solution of biotin conjugated mouse monoclonal antibodies to digoxin were added to 19.98ml of 0.01M phosphate buffered solution (pH 7.4), thoroughly mixed in a rotary shaker, then aliquotted into microtubes in quantities of 200 μ l.

30

9.10 Digoxin was conjugated with peroxidase according to a previously described protocol, see [21]. The resultant solution of peroxidase labelled digoxin (final concentration ~0.1mg/ml) was diluted with 0.01M phosphate saline buffer solution (pH 7.4) at a dilution ratio of 10 times then divided into aliquots of small volume (10 μ l) and stored at +4°C.

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9.11 Working buffer solution N°1 was made up as described in 4.11.

9.12 Working buffer solution N°2 was made up as 5 described in 4.12.

9.13 The sensing electrodes coated in polypyrrole film with bound streptavidin were removed from the storage buffer solution and each placed in a 10 microtube containing the solution of biotin conjugated mouse monoclonal antibodies to digoxin (from step 9.9) and incubated for 10 minutes at room temperature.

15 9.14 Simultaneously with step 9.13, 2 μ l of the solution of peroxidase labelled digoxin (from step 9.10) was added to each of the samples with known digoxin concentration (from step 9.8), having first removed 2 μ l from the sample.

20 9.15 On completion of steps 9.13 and 9.14, the sensing electrodes were transferred to the tubes containing peroxidase labelled and unlabelled digoxin (from step 9.14); the tubes plus sensing electrodes 25 were then placed in a thermomixer and held for 10 minutes at 37 \pm 1°C, mixing continuously.

30 9.16 On completion of 9.15, the sensing electrodes were removed from the microtubes, rinsed for 3-5 seconds in 0.01M PBS and each placed in a microtiter plate well filled with working buffer solution N°1.

35 9.17 The sensing electrode and reference electrode were connected to the electrical contacts

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of the holder connected to the PC-based measuring instrument, and the holder was positioned over the microtiter plate well filled with working buffer solution N°1 such that the sensing electrode and 5 reference electrode were immersed in the working buffer.

9.18 The custom software was started and used to record the sensing electrode potential in millivolts 10 relative to the reference electrode potential over a period of 30 seconds.

9.19 On completion of 9.18, the holder was positioned over a microtiter plate well filled with 15 working buffer solution N°2, in a manner similar to that described in 9.17.

9.20 The custom software was used to record the variation in millivolts of the sensing electrode 20 potential relative to the reference electrode potential over a period of 100 seconds.

9.21 Using the custom software, the area (integral) S_2 described by the curve of sensing 25 electrode potential variation versus reference electrode potential was calculated.

9.22 The operations described in 9.17-9.21 were repeated in sequence using the samples with known 30 digoxin concentration prepared at step 9.8.

9.23 Based on the results obtained at step 9.22, the custom software was used to plot the calibration curve " S_2 against digoxin concentration in the sample".

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Claims:

1. A sensing electrode for use in methods of electrochemical detection of an analyte, the sensing electrode comprising an electrically conductive electrode coated with a layer of electroconductive polymer with adaptor molecules selected from the group consisting of avidin, streptavidin, anti-FITC antibodies and a molecule capable of specifically binding to at least one class of receptor molecules immobilised therein or adsorbed thereto.

2. A sensing electrode as claimed in claim 1 in which the layer of electroconductive polymer has been doped with anions of large ionic radius.

3. A sensing electrode as claimed in claim 1 or claim 2 wherein the adaptor molecules are avidin or streptavidin and biotinylated receptors capable of binding said analyte are attached thereto via a biotin/avidin or biotin/streptavidin binding interaction.

4. A sensing electrode as claimed in claim 1 or claim 2 wherein the adaptor molecules are protein A or protein G and antibodies capable of binding said analyte are attached thereto via a protein A/antibody or protein G/antibody binding interaction.

5. A sensing electrode as claimed in claim 1 or claim 2 wherein the adaptor molecules are lectins and receptors capable of binding said analyte are attached thereto via a lectin/carbohydrate binding interaction.

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6. A sensing electrode as claimed in claim 1 or claim 2 wherein the adaptor molecules are anti-FITC antibodies and receptors capable of binding said analyte are attached thereto via a FITC/anti-FITC binding interaction.

5

7. An electrode assembly comprising a sensing electrode as claimed in any one of claims 1 to 6 and a reference electrode.

10

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20

8. A method of producing a sensing electrode for use in methods of electrochemical detection of an analyte, the sensing electrode comprising an electrically conductive electrode coated with an electroconductive polymer with adaptor molecules selected from the group consisting of avidin, streptavidin, anti-FITC antibodies and a molecule capable of specifically binding to at least one class of receptor molecules immobilised therein, the method comprising the steps of:

25

30

a) preparing an electrochemical polymerisation solution comprising monomeric units of the electroconductive polymer and adaptor molecules,

b) immersing the electrode to be coated in the electrochemical polymerisation solution, and

c) applying a cyclic electric potential between the electrode and the electrochemical polymerisation solution to coat the electrode by electrochemical synthesis of the polymer from the solution, said cyclic electric potential being applied for at least one full cycle.

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9. A method of producing a sensing electrode for use in methods of electrochemical detection of an analyte, the sensing electrode comprising an electrically conductive electrode coated with an 5 electroconductive polymer with adaptor molecules selected from the group consisting of avidin, streptavidin, anti-FITC antibodies and a molecule capable of specifically binding to at least one class of receptor molecules adsorbed thereto, the method comprising steps of:

a) preparing an electrochemical polymerisation solution comprising monomeric units of the electroconductive polymer,

15 b) immersing the electrode to be coated in the electrochemical polymerisation solution,

20 c) applying a cyclic electric potential between the electrode and the electrochemical polymerisation solution to coat the electrode by electrochemical synthesis of the polymer from the solution, said cyclic electric potential being applied for at least one full cycle; and

25 d) contacting the coated electrode with a solution comprising adaptor molecules such that the adaptor molecules are adsorbed onto the electroconductive polymer coating of the electrode.

30 10. A method as claimed in claim 8 or claim 9 wherein the adaptor molecules are avidin or streptavidin and the method further comprises the step of contacting the sensing electrode with a 35 solution comprising receptor molecules, said receptor

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molecules being conjugated with biotin such that said
biotinylated receptors bind to molecules of avidin or
streptavidin immobilised in or adsorbed to the
electroconductive polymer coating of the electrode
5 via a biotin/avidin or biotin/streptavidin binding
interaction.

11. A method as claimed in claim 10 wherein the
receptor molecules are monoclonal antibodies,
10 polyclonal antibodies, antibody fragments, antibody
mimics, chimaeric antibodies viral lysates,
recombinant proteins, synthetic peptides, hormones,
hormone receptors, single stranded nucleic acids, low
molecular weight molecules, chemical compounds
15 conjugated with proteins (haptens), fragments of
bacterial, plant or animal cells, lectins,
glycoproteins or carbohydrates.

12. A method as claimed in claim 8 or claim 9
20 wherein the adaptor molecules are protein A, protein
G or lectins.

13. A method as claimed in any one of claims 8
to 12 wherein the cyclic electric potential has a
25 sawtooth form.

14. A method as claimed in any one of claims 8
to 13 wherein the cyclic electric potential is
applied for at least two cycles.

30
15. A method as claimed in any one of claims 8
to 14 wherein the cyclic electric potential has a
peak value applied to the electrode which is less
than or equal to +2 volts.

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16. A method as claimed in any one of claims 8 to 13 in which a salt whose anions have a large ionic radius is added to the electrochemical polymerisation solution.

5

17. A method as claimed in claim 16 wherein the salt is sodium dodecylsulphate or sodium dextran sulphate.

10

18. A method as claimed in any one of claims 8 to 17 wherein the monomeric units of the electroconductive polymer are aniline, thiophene, furan or pyrrole.

15

19. A method of electrochemical detection of an analyte in a sample, which method comprises the steps of:

20

a) providing a sensing electrode having an electroconductive polymer coating, the coating having immobilised therein or adsorbed thereto receptors which are capable of binding the desired analyte to be detected in the sample;

25

b) contacting the sensing electrode with a test solution comprising the sample so that said desired analyte binds to said immobilised or adsorbed receptors;

30

c) contacting the sensing electrode with a solution comprising secondary receptors capable of binding to said analyte at a site spatially distinct from the site of binding to the immobilised or adsorbed receptors, said secondary receptors being conjugated with a charge label;

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5 d) monitoring the electric potential difference between the treated sensing electrode and a reference electrode when both are immersed in an electrolyte; and

10 e) monitoring the electric potential difference between the sensing electrode and a reference electrode following a change in the ionic strength of the electrolyte at constant pH.

15 20. A method of electrochemical detection of an analyte in a sample, which method comprises the steps of:

15 a) providing a sensing electrode having an electroconductive polymer coating, the coating having immobilised therein or adsorbed thereto receptors which are capable of binding to the desired analyte to be detected in the sample;

20 b) contacting the sensing electrode with a test solution comprising the sample so that said analyte binds to said immobilised or adsorbed receptors;

25 c) contacting the sensing electrode with a solution comprising competing molecules capable of binding to said immobilised or adsorbed receptors, said competing molecules being conjugated with a charge label;

30 d) monitoring the electric potential difference between the treated sensing electrode and a reference electrode when both are immersed in an electrolyte; and

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e) monitoring the electric potential difference between the sensing electrode and a reference electrode following a change in the ionic strength of the electrolyte at constant pH.

5

21. A method as claimed in claim 19 or claim 20 wherein the charge label has the following properties:

10 (i) it carries a net charge at the pH of the electrolyte of part d); and

(ii) the magnitude of this charge changes in response to a change in the ionic strength of the electrolyte at constant pH;

15 22. A method as claimed in claim 21 wherein the charge label is ferrocene, latex microspheres or gold.

20 23. A method as claimed in any one of claims 19 to 22 wherein steps (b) and (c) are performed simultaneously by contacting the sensing electrode with a test solution to which has been added secondary receptors or competing molecules conjugated with a charge label.

25

24. A method of electrochemical detection of an analyte in a sample, which method comprises the steps of:

30 (a) providing a sensing electrode having an electroconductive polymer coating, the coating having immobilized therein or adsorbed thereto receptors which are capable of binding to the desired analyte to be detected in the sample;

35 (b) contacting the sensing electrode with a

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test solution comprising the sample so that the said analyte binds to said immobilized or adsorbed receptors;

5 (c) contacting the sensing electrode with a solution comprising secondary receptors capable of binding to said analyte at a site spatially distinct from the site of binding to immobilized or adsorbed receptors, said secondary receptors being conjugated
10 with an enzyme;

15 d) monitoring the electric potential difference between the treated sensing electrode and a reference electrode when both are immersed in an electrolyte; and

20 e) monitoring the electric potential difference between the sensing electrode and a reference electrode following exposure to an electrolyte comprising the substrate for said enzyme.

25. A method of electrochemical detection of an analyte in a sample, which method comprises the steps of:

25 (a) providing a sensing electrode having an electroconductive polymer coating, the coating having immobilized therein or adsorbed thereto receptors which are capable of binding to the desired analyte to be detected in the sample;

30 (b) contacting the sensing electrode with a test solution comprising the sample so that the said desired analyte binds to said immobilized or adsorbed receptors;

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(c) contacting the sensing electrode with a solution comprising competing molecules capable of binding to said immobilized or adsorbed receptors, said competing molecules being conjugated with an
5 enzyme;

10 (d) monitoring the electric potential difference between the treated sensing electrode and a reference electrode when both are immersed in an electrolyte; and

15 (e) monitoring the electric potential difference between the sensing electrode and a reference electrode following exposure to an electrolyte comprising the substrate for said enzyme.

20 26. A method as claimed in claim 24 or claim 25 wherein the enzyme is capable of converting a substrate which directly affects the redox composition of the electroconductive polymer coating of the sensing electrode to a product which has no detectable effect on the redox composition of the said electroconductive polymer coating.

25 27. A method as claimed in claim 26 wherein the enzyme is a peroxidase.

30 28. A method as claimed in claim 24 or claim 25 wherein the enzyme is capable of converting a substrate which has no detectable effect on the redox composition of the electroconductive polymer coating of the sensing electrode to a product capable of directly or indirectly affecting the redox composition of the said electroconductive polymer
35 coating.

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29. A method as claimed in claim 28 wherein the product capable of indirectly affecting the redox composition of the electroconductive polymer membrane causes a change in the pH of the electrolyte of part 5 (e).

30. A method as claimed in claim 29 wherein the enzyme is a urease.

10 31. A method as claimed in claim 24 or claim 25 wherein the enzyme is capable of converting a substrate which has no detectable effect on the redox composition of the electroconductive polymer coating of the sensing electrode to a product which is a 15 substrate for a second enzyme, the action of the second enzyme generating a second product which directly or indirectly affects the redox composition of the electroconductive polymer coating of the sensing electrode.

20 32. A method as claimed in any one of claims 24 to 31 wherein steps (b) and (c) are performed simultaneously by contacting the sensing electrode with a test solution to which has been added 25 secondary receptors or competing molecules conjugated with an enzyme label.

30 33. A method as claimed in any one of claims 19 to 32 wherein the receptors capable of binding to the analyte to be detected are biotinylated and are attached to avidin or streptavidin immobilised in or adsorbed to the electroconductive polymer coating of the sensing electrode via a biotin/avidin or biotin/streptavidin binding interaction.

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34. A method as claimed in any one of claims 19
to 32 wherein the receptors capable of binding to the
analyte to be detected are antibodies and are
attached to protein A or protein G immobilized in or
5 adsorbed to the electroconductive polymer coating of
the sensing electrode via a protein A/antibody or
protein G/antibody binding interaction.

35. A method as claimed in any one of claims 19
10 to 32 wherein the receptors capable of binding to the
analyte to be detected contain a sugar moiety and are
attached to lectins immobilized in or adsorbed to the
electroconductive polymer coating of the sensing
electrode via a lectin/sugar binding interaction.

15 36. A method as claimed in any one of claims 19
to 32 wherein the receptors capable of binding to the
analyte to be detected are labelled with FITC and are
attached to anti-FITC antibodies immobilized in or
20 adsorbed to the electroconductive polymer coating of
the sensing electrode via a FITC/anti-FITC binding
interaction.

25 37. A method as claimed in any one of claims 19
to 36 wherein the sensing electrode is produced
according to the method of any one of claims 8 to 18.

30 38. A method as claimed in claim 33 wherein
steps (b) and (c) are performed simultaneously with a
step of contacting the sensing electrode with
biotinylated receptors by contacting the sensing
electrode with a test solution to which has been
added biotinylated receptors and secondary receptors
35 or competing molecules conjugated with a charge label
or an enzyme.

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39. A method as claimed in claim 19 wherein the secondary receptors are polyclonal antibodies or monoclonal antibodies.

5 40. A method as claimed in any one of claims 19 to 39 in which biological fluids such as whole blood, serum, lymph, urine, saliva, cerebrospinal fluid and semen are used as the test solution.

10 41. A method of electrochemical detection of an analyte in a sample, which method comprises the steps of:

15 (a) providing a sensing electrode comprising an electrically conductive electrode coated with a layer of electroconductive polymer with molecules of avidin or streptavidin immobilized therein or adsorbed thereto, said avidin or streptavidin molecules being attached to receptor molecule capable of binding the analyte to be detected attached via a 20 biotin/avidin or biotin/streptavidin binding interaction;

25 (b) contacting the sensing electrode with a test solution comprising the sample so that said desired analyte binds to said immobilized or adsorbed receptor molecules;

30 (c) monitoring the potential of the sensing electrode relative to a reference electrode when both are immersed in an electrolyte; and

35 (d) monitoring the potential difference of the sensing electrode relative to the reference electrode following a change in the ionic strength or composition of the electrolyte at constant pH.

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42. A method as claimed in claim 41 wherein the analyte to be detected is a nucleic acid and the receptor molecules are oligonucleotides.

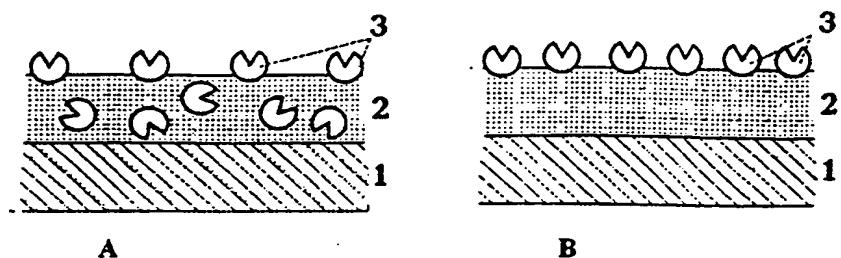


Fig.1

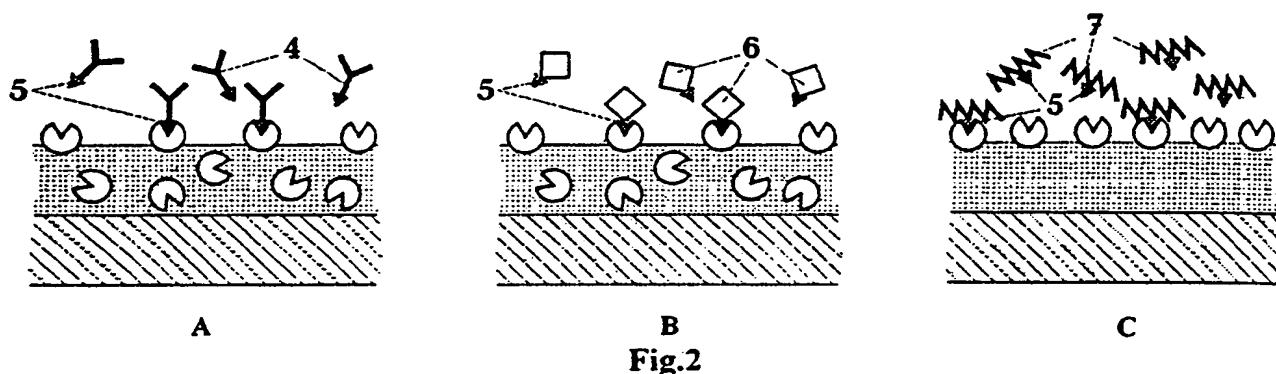


Fig.2

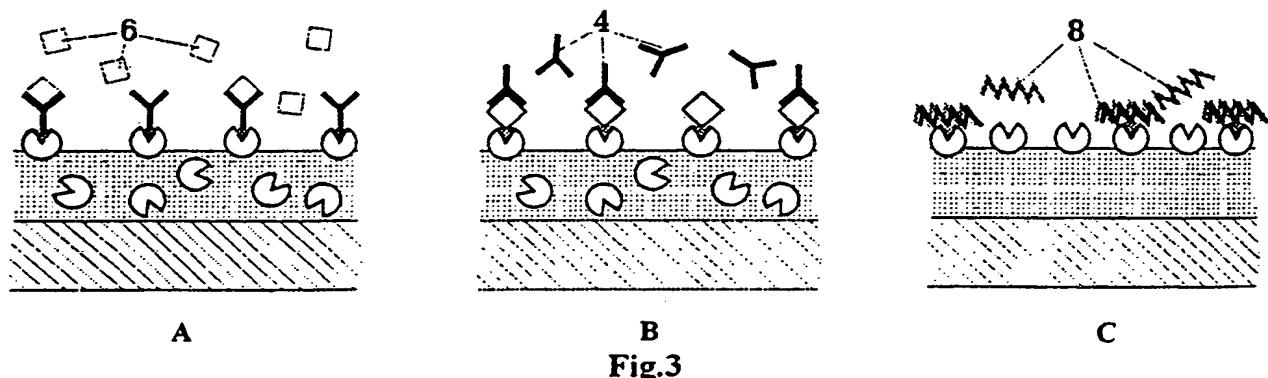


Fig.3

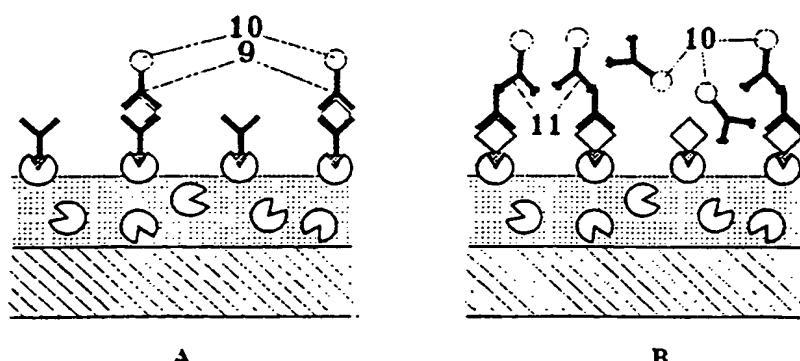


Fig.4

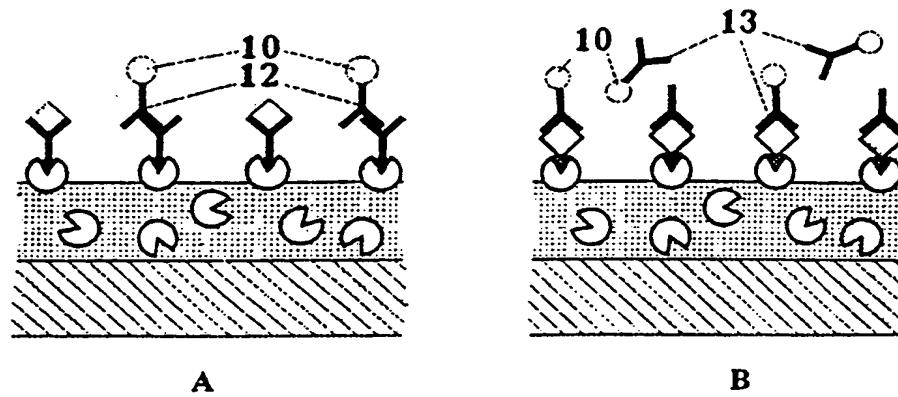


Fig.5

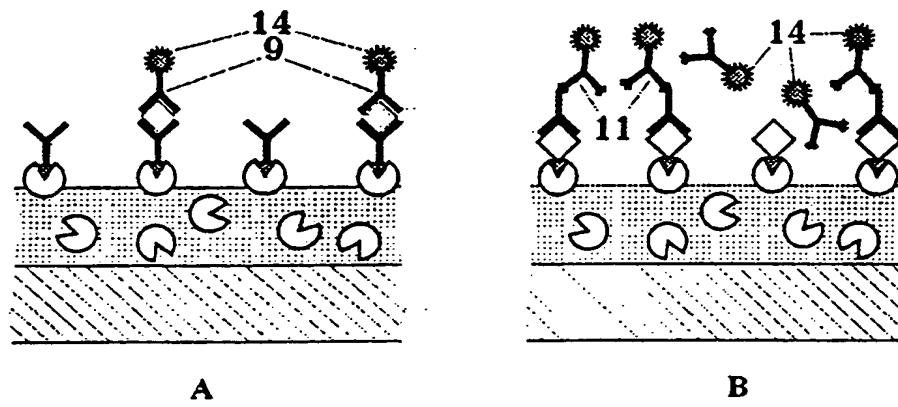


Fig.6

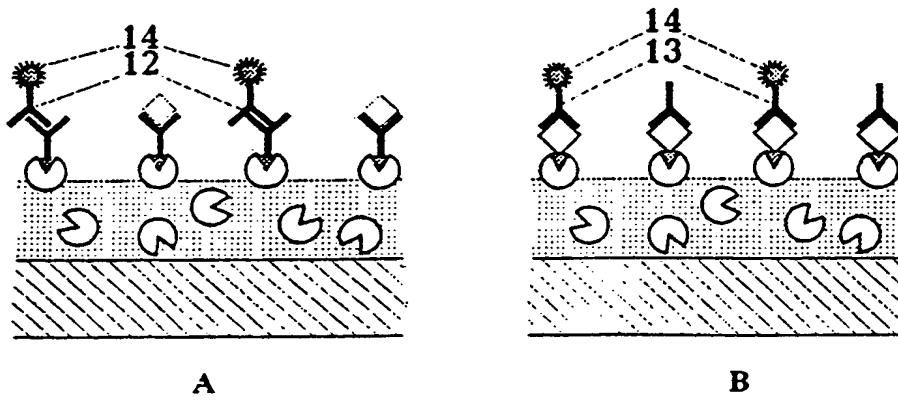


Fig.7

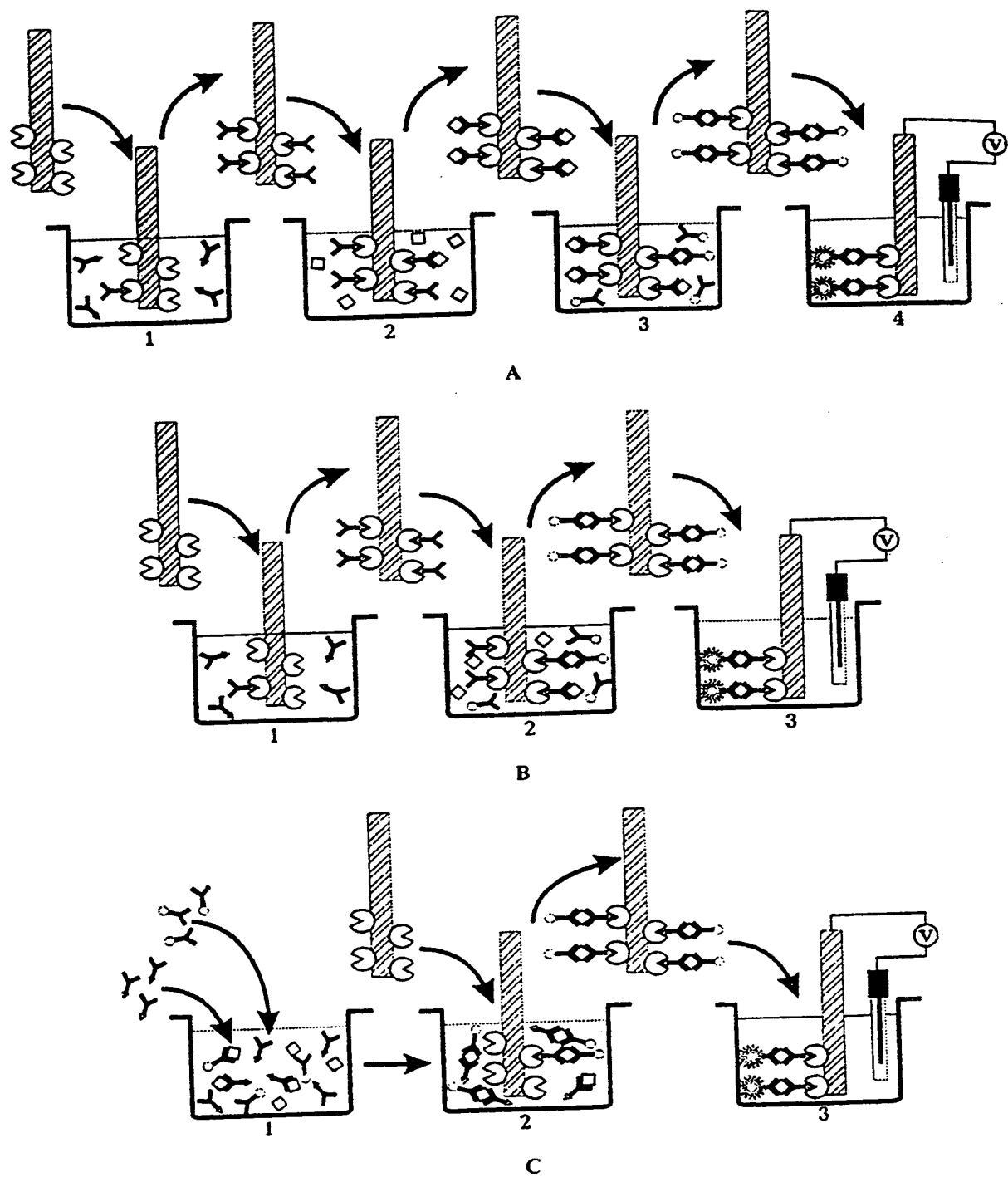


Fig.8

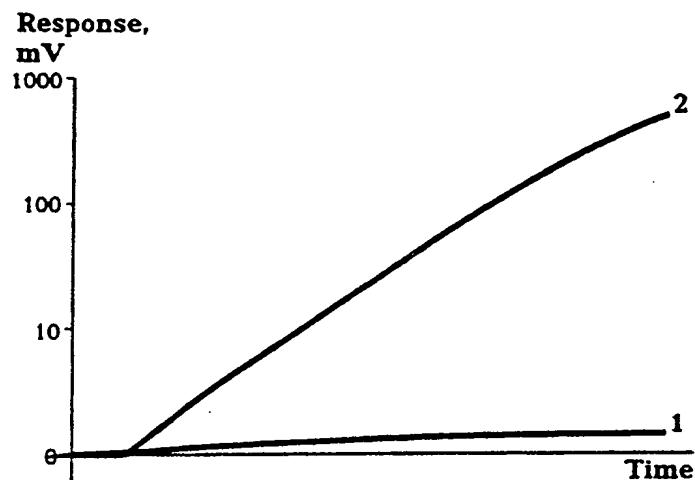


Fig.9

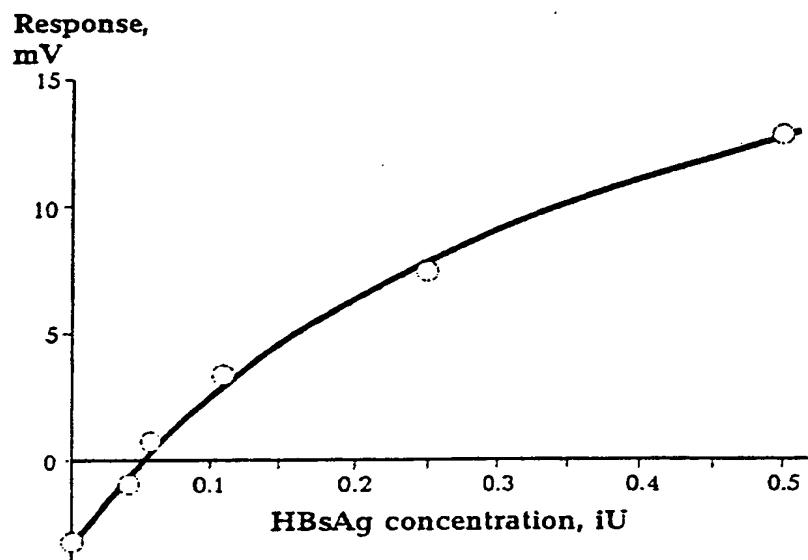


Fig.10

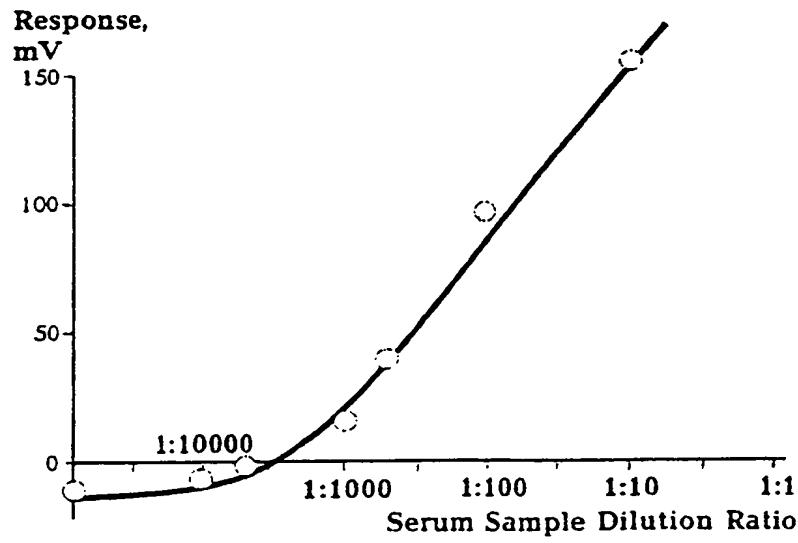


Fig.11

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02785

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/543 G01N27/327 C12Q1/28 C12Q1/58 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUISEPPi-ELIE, A. ET AL: "Electroconductive polymer thin films with internal bioactive moieties for biosensor applications" POLYM. MATER. SCI. ENG. (1995), 72, 404-5 , XP000853822 the whole document	1,8,9, 19,20, 24,25,41
A	WO 98 35232 A (NOVALON PHARMACEUTICAL CORP ;UNIV NORTH CAROLINA (US); FOWLKES DAN) 13 August 1998 (1998-08-13) examples	1,8,9, 19,20, 24,25,41
A	US 5 312 762 A (GUISEPPi-ELIE ANTHONY) 17 May 1994 (1994-05-17) abstract	1,8,9, 19,20, 24,25,41

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the International search

Date of mailing of the International search report

25 November 1999

03/12/1999

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Authorized officer

Moreno, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02785

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 89 11649 A (WOLLONGONG UNIADVICE) 30 November 1989 (1989-11-30) cited in the application the whole document	1,8,9, 19,20, 24,25,41
A	EP 0 193 154 A (CHEMO SERO THERAPEUT RES INST) 3 September 1986 (1986-09-03) cited in the application the whole document	1,8,9, 19,20, 24,25,41

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No

PCT/GB 99/02785

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US 5312762	A 17-05-1994	WO CA WO US US	9306237 A 2048692 A 9010655 A 5766934 A 5352574 A	01-04-1993 14-09-1990 20-09-1990 16-06-1998 04-10-1994
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